Vol. L *February, 1926* *No. 2*

BIOLOGICAL BULLETIN

THE PRECIPITIN REACTION IN THE STUDY OF ANIMAL RELATIONSHIPS.

ALAN ARTHUR BOYDEN,

ZOÖLOGICAL LABORATORY, UNIVERSITY OF WISCONSIN.

GENERAL HISTORICAL INTRODUCTION.

The precipitin reaction was discovered by Rudolph Kraus in 1897. Having injected a goat with sterile cholera culture filtrates, he found that the blood serum of the goat had come to possess the property of causing a precipitate to appear when it was mixed with perfectly clear solutions of cholera culture filtrates. Normal goat serum, of course, caused no such precipitate. Furthermore, the serum of the goat injected with cholera filtrates (cholera antiserum) caused no precipitate when mixed with bacterial filtrates other than cholera. Similar results were obtained when typhoid and plague culture filtrates were used for inoculation. In these cases typhoid antiserum caused a precipitate only in typhoid filtrates and plague antiserum only in plague filtrates. The reaction was therefore specific in that a precipitate occurred only when an antiserum was mixed with a filtrate of the particular kind of bacteria used for immunization. The substances developed in the blood serum of the immunized animal were called "precipitins" and from them the reaction got its name. The materials which were injected into animals for the purpose of inducing precipitin formation were called "precipitinogens."

The precipitin reaction became of general biological significance two years later when Bordet (1899) and Tchistovitch (1899) independently found that proteins other than bacterial could act as precipitinogens, *i.e.*, could induce the formation of pre-

cipitins when injected into animals. Bordet injected fowl blood into rabbits and rabbit blood into fowls and obtained specific antisera in either case. Tchistovitch injected eel serum and horse serum into rabbits and obtained likewise antisera which gave precipitins when mixed with the particular serum used for injection. From these and many similar results obtained by other workers it was learned that in general any foreign protein was capable of stimulating precipitin formation when injected into animals.

Now such a specific reaction might be applied to the solution of many biological and medical problems. It might be used to identify unknown bacteria, to diagnose an obscure disease, to detect the adulteration of meats, or to identify unknown blood stains in medico-legal cases. Indeed, the precipitin reaction has been used for all these purposes and for others as well. The amount of work done in the solution of these problems is considerable, and the literature dealing with them is extensive. It is desired here, however, to mention only the more important contributions to problems primarily zoölogical.

To Uhlenhuth belongs the greater part of the credit for having worked out a dependable method for the identification of unknown blood stains in criminal cases. But in these early studies, Uhlenhuth (1901) records results of importance to the zoölogist. It may be recalled that the precipitin reaction, as first known, was entirely specific. Uhlenhuth noted, however, that the reaction was not always strictly specific but that sometimes an antiserum against one kind of protein would react not only with this same protein (homologous reaction) but would also react, though to a less degree, with other closely related proteins as well (heterologous reaction). The precipitin reaction was thus proven to be not strictly specific though in fact it remained quantitatively so inasmuch as the homologous reaction was always stronger than any heterologous reaction with the same antiserum. Uhlenhuth then followed up this discovery with studies on the reactions of various egg albumins and pointed out the value of studies of this kind on related proteins.

By far the most important studies on related proteins have been made by Nuttall (1904). In his book "Blood Immunity

and Blood Relationship," Nuttall published the results of 16,000 tests made with thirty different antisera on 900 species of animal blood. Besides these qualitative tests there were included also 500 quantitative tests on various bloods. As a result of these tests Nuttall was able to state that the degree of reaction between an antiserum and various proteins was in proportion to the degree of relationship of these proteins to each other. Here, then, was a new method of measuring the "blood relationships" of animals, and the results obtained by Nuttall confirmed in general the existing classification of these animals based chiefly on comparative anatomy and embryology.

Since the outstanding works of Uhlenhuth and of Nuttall there have been many other contributions to the study of animal relationships based on precipitin tests. Hektoen, though interested primarily in the medico-legal aspects of the precipitin reaction, has published numerous results which have a direct bearing on problems of specificity and animal relationships. Following up the discovery of Uhlenhuth that the lens proteins of the Vertebrate eye are remarkably similar throughout, Hektoen (1922) has made many tests confirming Uhlenhuth's statements. Furthermore, Hektoen (Hektoen and Schulhof, 1924) has found that there are two antigenically distinct proteins in the crystalline lens and that apparently the lenses of all the chief groups of Vertebrates have these two elements in common. Hektoen (Hektoen and Welker, 1924) has also found that the blood serum of animals can be divided into several specific protein fractions. He has shown (Hektoen and Manly, 1923) that the semen of animals gives specific and group reactions, and that the same is true of their hemoglobins (Hektoen and Schulhof, 1923). Throughout his work he has repeatedly succeeded in obtaining specific precipitin reactions. The milk of mammals has been shown to give much the same results in the study of relationships as the blood serum.

In all this work there has been a fair degree of uniformity in results obtained. But there have been many other studies of more uncertain value. Dervieux (1921) in working with an anti-human semen serum claimed to have found an individual specificness to the reaction. Geyer (1913) states that he could distinguish

between male and female Lepidopteran gonads by means of this test. Many other attempts have been made to show an organ specificity for the precipitin reaction but so far all this work has failed of confirmation, except in the case of the lens of the Vertebrate eye.

As a matter of fact even the results of Uhlenhuth, Nuttall, and Hektoen have not gone unchallenged. Strube (1902), Kister and Wolff (1902), and many others claimed that the precipitin reaction was not always specific even in the quantitative sense. And in more recent years similar claims have again been made. Thus Friedberger and Collier (1919) state that an antiserum against one kind of protein may react with equal or even greater intensity with non-related proteins. A year later Friedberger and Jarre (1920) reported upon "aspecific antisera" and claimed that many of the antisera which they produced reacted without regard to the relationship of the proteins used. Reeser (1919) independently in Holland claims the same thing and is confirmed by Yu (1923) and again by Friedberger and Meissner (1923) in Germany.

Thus the matter stands to-day, the value of the precipitin reaction in the study of animal relationships being by no means completely established. For if the precipitin reaction does not take place according to the relationship of the proteins tested, as Friedberger claims, its results will always be of uncertain value in such studies.

One more problem that has been attacked with the aid of immune reactions including the precipitin test should be mentioned. This is in the field of experimental evolution and the work referred to is that of Guyer and Smith (1920). By injecting antisera against rabbit lens into pregnant rabbits these investigators have obtained hereditary eye defects which appeared in many succeeding generations. Furthermore, just recently Guyer (1925) has been able to demonstrate that by needling the lens of rabbits lens defects may be caused in their young. The significance of these discoveries in the solution of problems of experimental evolution may be almost unlimited.

The precipitin reaction has then contributed something to the solution of at least two fundamental zoölogical problems,

viz., animal relationships and evolution. It would seem perhaps worth while to make a careful study of the reaction in the hope of clearing away discordant results and of properly evaluating the reaction as a research tool. And inasmuch as precipitins are little known to zoölogists in general it has seemed advisable to explain the reaction more fully than would otherwise be necessary.

PURPOSE OF THIS INVESTIGATION.

The purposes of this investigation are (1) to study the accuracy and the dependability of the precipitin reaction and factors which may increase these qualities, (2) to apply an improved technique of the reaction with a known per cent. of error to the study of animal relationships.

TECHNIQUE.

Historical Review.

As first performed by Kraus in 1897 the precipitin reaction consisted in the mixing of a sterile antiserum (formed by injecting goats with bacterial culture filtrates) with clear sterile bacterial filtrates in small glass tubes which were then placed in the incubator at 37° C. After twenty-four hours the tubes were examined and those which were markedly cloudy and showed flocculent precipitates were recorded as positive. The "titer" or strength of any antiserum was measured by putting into a series of similar tubes containing increasing dilutions of the antigen (protein to be tested) in normal salt solution, a definite amount of antiserum, then mixing, incubating, and recording after a certain definite time the highest dilution in which a precipitate occurred. As controls, tubes containing normal blood serum and antigen dilution, and tubes containing antiserum plus salt solution alone, were used.

This technique was used by many workers in the titration of a great variety of antisera and antigens. It soon came to be noticed, however, that the end point of this "flocculation" test was exceedingly hard to determine, for a cloudiness might occur in the tubes without a settled precipitate and therefore the cloudiness of the experimental tubes graded off imperceptibly

into the cloudiness of the controls. The probability of error in such determinations must have been very high and no doubt some of the discordant results obtained by early investigators were due to this very fact.

Great steps in advance came with the works of Ascoli (1902) and Fornet and Muller (1908, 1910) in the development of the "ring test" and with Nuttall's quantitative measurements of the amount of precipitate formed (1902). Ascoli suggested that the antiserum be carefully layered under the antigen dilution somewhat as the Heller's nitric acid test is performed. Then in positive reactions a definite white stratum of precipitate forms at the zone of contact between antiserum and antigen, the so-called "ring," which is quite obvious and gives the reaction a sharp end point. Fornet and Muller further studied this technique and recommended this ring test strongly because of its greater definiteness. Today the best workers employ this test and indeed there is no longer any excuse for the continued use of the older flocculation test.

Nuttall published methods for making a quantitative flocculation test in 1902. His method consisted in the volumetric measurement of the amount of precipitate formed as a result of mixing a definite amount of an antiserum with a definite amount of a certain antigen dilution. Repeated tests on homologous bloods gave an error of about ten per cent. but sometimes when testing an antiserum with a heterologous blood the error reached fifty per cent. This error seems due partly to the small amounts of precipitate measured and partly to variations in the closeness with which the particles of the precipitate became packed.

The effects of temperature and of salt concentration on the titer of antisera have also been studied. Though there were some discordant results the influence of temperature was found to be well marked, the rate of precipitation increasing with temperature up to about 40° C. With regard to the effect of salt concentration there is less agreement. In general, however, increasing salt concentration decreases the amount of precipitate. Normal physiological salt solution (.85 to .9 per cent.) is generally used today. Hektoen (1918) recommends a salt solution of double the strength of normal saline for use with chicken antisera. This he states increases the specificity of the antiserum.

Recently the effect of variations in the H-ion concentration of the salt solution on the reaction has been studied. Using the flocculation test Mason (1922) found no significant effect within a range of P_H 4.5 to 9.5. However the flocculation test is not sensitive enough to show a slight effect had there been one. Evans (1922) and Mason and Sanford (1924) find that the H-ion does have an effect on various serological reactions and recommend buffered physiologic salt solutions for use in serological work.

With regard to the mode of injection of animals it has generally been found that intravenous injections give higher titers than any others. Such injections are given in increasing doses as four, six, eight cubic centimeters of serum on successive days or at two to five day intervals. The titer reaches its climax about the tenth day after the last injection. Rabbits and chickens have been found to be the most practical precipitin producers.

Material and Methods Used in this Investigation.

Rabbits and chickens have been used to produce the antisera. They have been injected with sterile blood sera of healthy sheep, goat, pig, horse and dog. The injections were usually intravenous though many were intraperitoneal. They were given for the most part at three day intervals the dosage being 4 cc., 6 cc., and 8 cc. of the particular blood serum used as antigen. Ten days after the third injection the rabbits were bled from the median ear artery and the fowls from the wing vein, a five cc. sample being withdrawn for a preliminary titration. In many cases the antisera so obtained possessed a titer of 6,400 or over. That is, a definite ring appeared in a dilution of one part of antigen in 6,400 parts of salt solution, at 1 hour, but did not appear in any greater dilutions. In other cases one or two more injections were given, the first one being 2–4 cc. and given intraperitoneally, the second 5–10 cc. and given intravenously. No symptoms of anaphylaxis have ever been noted. Usually the titer of these reinjected animals had now increased to 6,400 or over. All such titers were thought to be satisfactory and the animals possessing them were bled completely as soon as possible

after the preliminary titration. Both rabbits and fowls were starved for eighteen hours preceding the final bleeding in order that the blood be as clear as possible.

The rabbits were bled from the dorsal aorta. The animals were first anæsthetized with ether and then a median ventral incision was made. The viscera were moved to one side and the dorsal aorta carefully cleaned of fat and connective tissue for a short distance. The aorta was then clamped off centrally and distally and a sterile canula inserted through a small incision and securely ligatured. The central clamp was then removed and the blood allowed to flow through sterile rubber tubing into centrifuge tubes. In this manner 75–100 cc. of blood were usually obtained.

Fowls were bled completely after decapitation, the amount of blood varying from 35–125 cc. depending upon the size of the individual.

The blood was allowed to stand for two or more hours in the ice-box after which time the clot was broken up and the blood centrifugalized. The antiserum was then poured off and stored in 5 cc. sterile, rubber-stoppered vaccine vials which were then kept in the ice-box. In many cases the antisera were filtered through small Berkfeld filters before bottling to insure sterility.

The blood sera used as antigens were obtained from healthy animals. The beef and pig bloods were obtained from the Mayer Packing Plant, the horse blood from Mr. Fred Rieder, and the sheep and goat bloods from animals kept by the Department of Zoölogy. Besides these, other bloods were used for titration only. The latter included human, rat, guinea pig, rabbit, fish (carp) and fowl. The sera were obtained after clotting and centrifugalizing and were usually filtered through Berkfeld filters before storing in 5 cc. ampules in the ice-box.

From these blood sera standard solutions were made. These standards were usually 2 or 4 per cent. dilutions of a given serum in normal saline (sometimes 10 per cent.). The standards were made in 100 cc. volumetric flasks and filtered through a Berkfeld filter and then stored in the usual manner in 5 cc. vials. The concentration of protein in each standard was determined by making modified Kjeldahls on 5 or 10 cc. samples

according to the method of Folin and Wright (1919). Total nitrogen was determined on one sample and non-protein nitrogen on another, the protein in the latter being removed by precipitation by sodium tungstate (10 per cent.; 1 cc.) and sulphuric acid (2/3 N; 1 cc.). The protein nitrogen was then obtained by difference and the protein calculated (protein N x 6.25) as grams per 100 cc. serum. The titrations recorded hereinafter have all been made with these standard antigen solutions of known protein concentration.

The ring test has been employed throughout, the observations being made at room temperature. As suggested by Evans (1922) buffered salt solutions have been made according to Sorenson's tables given in Clark (1922). These buffered solutions were made to have a P_H of 7 determined colorimetrically except in certain cases where the P_H has been specially noted. The successive increasing dilutions have been made according to the usual Stern-Korte method. Thus a series of small clean glass tubes is set up in a rack and to each tube is added ½ cc. of buffered salt solution with a sterile 10 cc. pipette. To the first tube of the series is added ½ cc. of a standard antigen dilution using a 1 cc. pipette. The fluids are mixed by sucking up into this pipette several times. From this mixture (now ½ as concentrated as the standard solution) ½ cc. is transferred to the next tube, etc. Thus each tube in the series contains a concentration of protein in buffered salt solution (.85 per cent. NaCl for rabbit sera and usually 2.25 per cent. for chicken sera) one half as great as the preceding tube. The antisera used were generally of a strength of about 1 : 10,000 which requires eight tubes beginning with a 2 per cent. standard solution. Now to each of these dilutions beginning at the highest is added .1 cc. of the antiserum to be titrated the pipette being placed in the bottom of the tube and the antiserum carefully layered under the antigen dilution. As a control .1 cc. of the same antiserum is layered under ½ cc. of buffered salt solution. The tubes are then allowed to stand at room temperature and read at 20, 40, and 60 minutes by light from an electric bulb transmitted through a narrow horizontal slit behind the tubes.

When a suitable antiserum has finally been obtained pre-

liminary titrations are first made with the homologous antigen and then with various heterologous antigens in the manner just described. But in all cases in which sufficient antiserum was available these preliminary titrations were followed by one or more double titrations made in the following manner.

One series of tubes was set up with dilutions starting from a 2 per cent. standard solution. A second series was then set up starting with a dilution of 1 : 150 of this same antigen. This was made by mixing ½ cc. of 2 per cent. solution (1 : 50) with 1 cc. of salt solution. By using this double series of tubes the difference between the concentration of one tube and its nearest successor was reduced to 50 per cent. instead of 100 per cent. See Fig. I.

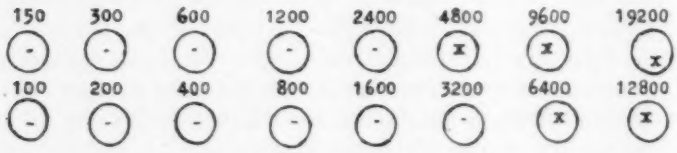


FIGURE 1. A diagram to illustrate the double series of dilutions employed in titration.

Now if the preliminary titration had given a titer of 6,400, in the final titration only the tubes from 4,800 to 19,200 (marked with an *x* in Fig. 1) would be given antiserum. The reading in the final titration falls generally within one tube of the preliminary titration. By using this double titration a more accurate value of the titer is obtained without the need of using excessive amounts of antiserum. The tubes containing antiserum were then put in order of increasing dilution in a rack and read as described.

EXPERIMENTAL RESULTS.

RESULTS ON TECHNIQUE.

1. *The Range of Error in Reading the Ring Test.*

Experimental results of any kind whatever have significance only insofar as it is definitely known that they pass beyond the range of experimental error. The first step in the application of the precipitin reaction to biological problems should then consist in the establishment of the range of error in reading the reaction for each investigator.

Rabbit P41 was injected with goat serum. The antiserum so obtained was repeatedly titrated against standard goat antigens at a P_H of 7. A double series of dilutions was made so that the succeeding tubes in the series had concentrations of antigen 50 per cent. less than their predecessors. The results of twelve such titrations are shown in Table I.

TABLE I.

RANGE OF ERROR IN READING THE RING TEST.

Antigen 2 Per Cent. Goat Serum, Antiserum P41.

Number of Test.	Order of Tubes.	4,800.	6,400.	9,600.	12,800.	Time.
1	Known	+	−	−	−	20 minutes
2	"	+	−	−	−	20 "
3	"	+	+	−	−	20 "
4	"	+	+	−	−	20 "
5	"	+	+	−	−	20 "
6	"	+	+	−	−	20 "
1	"	+	+	−	−	40 "
2	"	+	+	−	−	40 "
3	"	+	+	+	−	40 "
4	"	+	+	+	−	40 "
5	"	+	+	+	−	40 "
6	"	+	+	+	+	40 "
1	"	+	+	+	−	60 "
2	"	+	+	+	+	60 "
3	"	+	+	+	+	60 "
4	"	+	+	+	+	60 "
5	"	+	+	+	+	60 "
6	"	+	+	+	+	60 "
1	Unknown	+	−	−	−	20 "
2	"	+	−	−	−	20 "
3	"	+	+	−	−	20 "
4	"	+	+	−	−	20 "
5	"	+	+	−	−	20 "
6	"	+	+	−	−	20 "
1	"	+	+	−	−	40 "
2	"	+	+	−	−	40 "
3	"	+	+	+	−	40 "
4	"	+	+	+	−	40 "
5	"	+	+	+	−	40 "
6	"	+	+	+	−	40 "
1	"	+	+	+	−	60 "
2	"	+	+	+	−	60 "
3	"	+	+	+	−	60 "
4	"	+	+	+	−	60 "
5	"	+	+	+	+	60 "
6	"	+	+	+	+	60 "

Table showing 12 titrations of the same antiserum and antigen using a double series of tubes and dilutions.

The results shown in Table I. are (1) that readings of the ring test generally corresponded at a given period, (2) that the titer increased with time up to an hour, (3) that the greatest deviation in the reading was plus or minus one tube or 50 per cent. in terms of titer, (4) that when the order of the tubes was that of increasing dilutions and known to the reader a higher reading was usually obtained than when the order was irregular and unknown. This latter result is natural for if the tubes are arranged in order of increasing dilutions of antigen, the rings occur strongly in the lower dilutions and gradually less strongly in the higher dilutions and the first tube to be apparently negative often on close observation will be seen to possess a faint but definite ring. This might easily be overlooked if the order of the tubes is unknown. For this reason the order of the tubes has been known in all titrations on relationships.

Now a possible error of plus or minus 50 per cent. in reading the ring test (which increases to 100 per cent. when a single series of dilutions is used) doubtless seems very high but one must remember that this is a biological test and that in spite of this error proteins may be identified in dilutions far beyond the range of any chemical method known at present. By "possible error" is meant simply that inasmuch as the dilutions tested were each 50 per cent. less than the preceding dilution, the maximum error, should the end point actually fall somewhere between two successive dilutions, would be 50 per cent. It is to be remembered that this possible error is inherent only in the serological titration not in the preparation of the antigen solution (error less than 4 per cent. in nitrogen determinations). The probable error would not be more than half as great as this possible error. It is felt that the use of a double series of dilutions is justified on account of the more accurate reading thus obtained. A triple or quadruple series of dilutions might be used with an even greater accuracy but the labor involved in their use is so great as to preclude their practice.

2. *The Sensitivity of the Ring Test.*

Fifteen antisera have been carefully titrated with standard antigens. Their homologous titers at one hour were distributed as follows:

Titer or Dilution	4,800	9,600	12,800	19,200	25,600	76,800	102,400	204,800
No. of Antisera.....	1	1	3	3	4	1	1	1

The concentration of antigen in these dilutions being known, it is possible to calculate the amount of protein present in the last tube in which a positive reaction occurred. The antiserum with a titer of 4,800 positively identified the presence of .0088 mgms. of goat serum. The most powerful antiserum produced identified sheep serum when less than .0002 mgms. of protein was present. The other antisera fell in between these limits. Needless to say even the weakest antiserum employed was far more sensitive to a specific protein than any chemical method known. Much of the value of the precipitin reaction in research is based on this great sensitivity.

TABLE II.

THE EFFECT OF ANTIGEN CONCENTRATION ON TITER.

Time.	Antigen.	3,200.	4,800.	6,400.	9,600.	12,800.
20 minutes...	1%	+	−	−	−	−
20 " ...	1%	+	−	−	−	−
20 " ...	1%	+	−	−	−	−
20 " ...	1%	+	−	−	−	−
20 " ...	2%	+	+	−	−	−
20 " ...	2%	+	+	−	−	−
20 " ...	2%	+	+	+	−	−
20 " ...	2%	+	+	+	−	−
20 " ...	2%	+	+	+	−	−
20 " ...	2%	+	+	+	−	−
40 " ...	1%	+	−	−	−	−
40 " ...	1%	+	+	−	−	−
40 " ...	1%	+	+	−	−	−
40 " ...	1%	+	+	−	−	−
40 " ...	2%	+	+	+	−	−
40 " ...	2%	+	+	+	−	−
40 " ...	2%	+	+	+	+	−
40 " ...	2%	+	+	+	+	−
40 " ...	2%	+	+	+	+	−
40 " ...	2%	+	+	+	+	−
60 " ...	1%	+	+	−	−	−
60 " ...	1%	+	+	−	−	−
60 " ...	1%	+	+	−	−	−
60 " ...	1%	+	+	+	−	−
60 " ...	2%	+	+	+	+	−
60 " ...	2%	+	+	+	+	−
60 " ...	2%	+	+	+	+	−
60 " ...	2%	+	+	+	+	−
60 " ...	2%	+	+	+	+	+
60 " ...	2%	+	+	+	+	+

3. *The Effect of Antigen Concentration on the Titer of Antisera.*

Table II. shows the result of titrating the same antiserum (P41) with two homologous standard antigens of different strengths. All tubes were unknown in this experiment.

The results given in Table II. show that the titer of an antiserum varies directly with the concentration of the antigen used. Indeed it has long been known in a general way that such was the case. It has also been known that various antisera differ in titer when tested with the same homologous antigen. There are therefore two chief factors in this reaction, (1) the concentration of antigen, (2) the concentration of antibody in the antiserum. Now of these two the latter is but partly controllable. For we may inject various amounts of protein into animals but we must take whatever titer of antibody they yield. On the other hand the concentration of antigen can be definitely controlled and this should be made the standard of comparison for all workers in this field. For this reason only standard antigen solutions have been used in this investigation.

The results of the nitrogen determinations on the standards used are given in Table III.

From the data given in Table III. it may be seen: (1) that the blood sera used in general possessed about the same protein concentration, but that the dog serum was enough lower than the average to affect definitely the titer of its reactions, (2) that the amount of non-protein nitrogen in the fresh blood sera used was generally so small as to be well within the range of error of the titration, (3) that the amount of non-protein nitrogen generally increased in the standard solutions which were sometimes kept for several months. The values for non-protein nitrogen in these older standards sometimes approached a level high enough to become of consequence in the comparative titration of heterologous antigens.

Probably in fresh sera the error due to calculation of protein from total nitrogen would seldom exceed 10 per cent. Total nitrogen determinations alone would then give figures sufficiently accurate for comparative studies. It is hoped that the users of the precipitin reaction in the study of related proteins or in other quantitive studies will feel the necessity of getting com-

parable results and will come to make total nitrogen determinations on all antigens used in titrations. Perhaps it would be well to speak of the latter as secondary antigens to distinguish them from proteins injected into animals for the purpose of inducing antibody formation which would then be called primary antigens.

TABLE III.

NITROGEN DETERMINATIONS ON STANDARD SOLUTIONS.

Standard.	Date of Preparation.	Total N Grams per 100 cc. Serum.	Date of Determination.	Non-protein N in mgms. per 100 cc. Serum.	Date of Determination.	Protein in gms. per 100 cc. Serum.	% of Non-protein N in Total Nitrogen.
Beef serum							
No. 1–2%..	1– 6–25	1.295	1– 7–25	51	1–13–25	7.736	4.4
" 2–2%..	1–18–25	1.365	1–29–25	10	1–29–25	8.468	.74
" 3–2%..	4–20–25	1.176	4–28–25	trace	4–28–25	7.350	
Dog serum							
4%.....	2–23–25	.805	5– 1–25	119	5– 1–25	4.290	14.8
Goat serum							
No. 1–2%..	1–11–25	1.2859	1–13–25	87.5	1–13–25	7.487	7.4
" 2–2%..	3–24–25	1.120	4–28–25	140	4–28–25	7.925	12.5
Horse serum							
4%.....	2–23–25	1.085	5– 1–25	61.25	5– 1–25	6.394	5.6
Human serum 2%..	1–19–25	1.260	1–19–25	32.9	1–19–25	7.668	2.6
Pig serum							
No. 1–2%..	1– 6–25	1.309	1– 7–25	145.25	1–14–25	7.275	11.0
" 2–4%..	2–27–25	1.185	5– 1–25	94.5	5– 1–25	6.812	8.0
Sheep serum							
No. 1–2%..	1– 6–25	1.232	1– 7–25	96.25	1–13–25	7.100	7.7
" 2–2%..	3– 8–25	1.330	5– 1–25	140.0	5– 1–25	7.438	10.9
Tame rat							
2%.....	1– 4–25	1.575	5– 1–25	49.7	5– 2–25	9.097	7.7

Now when proteins other than fresh sera are used as secondary antigens, the necessity of determining their concentration is very much greater. For the concentration of protein in the sera of animals which have died of various diseases (so often used by Nuttall), or in extracts of filter paper soaked in blood and then dried, or in extracts of blood stains from various objects, or in extracts of tissues, varies exceedingly. This it seems was the chief source of error in Nuttall's work as it has also been in the work of many others. For heretofore nothing but a foam test, or a crude nitric acid boiling test have been used

in the determination of antigen concentration. The precipitin reaction seems worthy of better technique than this.

4. *The Effect of the Time of the Reading on Specificity.*

Since the titers (both homologous and heterologous) of fifteen antisera have been recorded at twenty, forty, and sixty minute intervals, the effect of time on the specificity of the reactions can be shown. The results are given in Table IV.

TABLE IV.

RELATIVE SPECIFICITY OF READINGS AT 20, 40, AND 60 MINUTES.

Anti-serum.	Antigen.	Heterologous Reactions in % of Homologous Reaction.		
		20 min.	40 min.	60 min.
A21 anti-pig	Pig	100 %	100 %	100 %
	Beef	12.5	8.3	12.5
	Horse	6.6	8.3	9.4
	Goat	6.6	6.6	6.25
	Sheep	4.7	6.6	6.25
	Dog	4.7	6.6	4.7
	Human	2.4	2.1	3.13
A24 anti-beef	Beef	100	100	100
	Sheep	37.5	50.0	50.0
	Goat	37.5	50	50
	Pig	9.4	25	16.6
	Horse	12.5	25	25
	Dog	9.4	18.8	16.6
	Human	12.5	18.8	12.5
P40 anti-sheep	Sheep	100	100	100
	Goat	66	100	75
	Beef	33.3	50	50
	Pig	1.04	2.08	3.1
	Horse	0.52	2.08	3.1
	Dog	1.04	2.08	3.1
	Human	0.52	0.52	0.58

The three antisera whose reactions are given in Table IV. have been taken at random from the entire number and are believed to be typical of them all. Of the eighteen heterologous reactions recorded fourteen showed a greater specificity at twenty minutes than at forty minutes or one hour. That is, the heterologous reactions at twenty minutes were proportionally less compared with the homologous reactions at the same time than at any later time. Those facts are of significance, for in medico-

legal work where the greatest possible specificity is desired the earlier reading is better, whereas in the study of relationships a later reading is preferable. Experience has shown that one hour is the latest time at which the ring test may be read satisfactorily at room temperature. After that time the rings become diffuse and tend to disappear.

5. *The Effect of P_H on the Titer of an Antiserum.*

An antibeef serum (A8) was titrated at several different P_H values as shown in Table V. The buffered solutions were made according to Sorenson's tables as recommended by Evans (1922) and were tested colorimetrically after sterilization in the autoclave. The homologous readings at one hour are given in the table.

TABLE V.

THE EFFECT OF P_H ON TITER.

P_H.	3,200.	4,800.	6,400.	9,600.	Control.
5.5.....	+	±	±	±	±
5.5.....	+	±	±	±	±
6.0.....	+	−	−	−	−
6.0.....	+	+	−	−	−
6.0.....	+	+	+	−	+
6.0.....	+	+	±	±	±
7.0.....	+	−	−	−	−
7.0.....	+	−	−	−	−
7.0.....	+	+	−	−	−
7.0.....	+	+	−	−	−
8.0.....	+	−	−	−	−
8.0.....	+	−	−	−	−

From the data given in Table V. it may be seen that a spontaneous precipitate often appeared in the controls below a P_H of 7. The titer of the antiserum was not affected in any marked way except through the effect on the control. Such a spontaneous precipitate in the control must be prevented and this alone justifies the use of buffered solutions in the precipitin test. Other workers claim even greater effects of the P_H on serological reactions.

6. *The Effect of Sodium Chloride Content on Titer.*

The titrations of rabbit antisera have all been made with .85 per cent. buffered saline with a P_H of 7 except in special

experiments. It was noted, however, that certain chicken antisera gave spontaneous rings in such salt solutions. It became necessary then to inhibit this reaction in order to record ring tests due to the presence of antigen in the salt solution. Following a suggestion of Hektoen's (1918) a more concentrated salt solution was used. It was found that in some cases a 2.25 per cent. salt solution was necessary to inhibit completely this spontaneous ring. The question then arose as to whether such a heavier salt concentration would alter the group reactions of a given antiserum. To settle this matter a chicken antiserum which did not require a 2.25 per cent. saline was nevertheless titrated with the same series of antigens in both .85 per cent. and 2.25 per cent. saline. The results are shown in Table VI.

TABLE VI.

THE EFFECT OF NaCl CONTENT ON TITER.

Antiserum.	Salt Concentration.			
	.85% Titer.	P_H 7 Per Cent.	2.25% Titer.	P_H 7 Per Cent.
C8 (antibeef) Beef.....	102,400	100 %	25,600	100 %
Sheep....	102,400	100	12,800	50
Goat.....	102,400	100	12,800	50
Pig.....	12,800	12.5	800	3.1
Horse....	6,400	6.25	800	3.1
Dog.....	3,200	3.1	800	3.1
Human...	3,200	3.1	400	1.6

Table VI. shows the distinct inhibition due to the heavier salt solution. Furthermore the specificity of the antiserum has been increased. As a matter of fact the range of reactions in chicken antisera is more closely parallel with that of rabbit antisera when this heavier salt solution is used (see Figs. 3 and 5).

7. *Effect of Filtration on Titer.*

When an antiserum becomes contaminated it must be filtered at once for the titer rapidly decreases during putrefaction. The possible effect of such filtration on the specificity of reaction of antisera must then be known. For this purpose parallel titra-

tions have been made on both rabbit and chicken antisera before and after filtration. The results are shown in Table VII.

The results given in Table VII. are typical of them all with the exception of a single chicken antiserum which apparently lost some of its antibodies on filtration. Small Mandler filters

TABLE VII.

THE EFFECT OF FILTRATION ON TITER.

Antiserum.	Antigen.	Unfiltered.		Filtered.	
		Titer.	Per Cent.	Titer.	Per Cent.
P123 (antidog).....	Dog.....	19,200	100 %	25,600	100 %
	Beef....	800	4.7	1,200	4.7
	Horse...	400	2.4	600	2.4
	Sheep...	200	1.2	600	2.4
	Goat....	200	1.2	400	1.6
	Pig.....	100	.6	200	.8
	Human..	100	.6	400	1.6
C17 (antisheep).....	Sheep...	51,200	100	76,800	100
	Beef.....	19,200	37.5	25,600	33.3
	Horse...	800	1.56	800	1.04
	Pig.....	000	0.00	200	.26
	Dog.....	100	.2	100	.13
C11 (antisheep).....	Sheep...	19,200	100	25,600	100
	Goat....	12,800	66.66	25,600	100
	Beef.....	6,400	33.33	19,200	75
	Dog.....	1,600	8.33	3,200	12.5

have been used for this filtration. In general there has been no loss of titer. In fact the titer usually increases after filtration due to the increased clarity of the antiserum and the consequent greater ease of detecting a fine ring in the last tube. Insofar as the effect on specificity has passed beyond the range of error it has been in the direction of a lesser specificity and would thus tend to neutralize the effect of using 2.25 per cent. salt solutions in the chicken antisera.

RESULTS ON RELATIONSHIPS.

The experimental results bearing on matters of technique have all been secondary to the main problem of this investigation, viz., the application of the precipitin reaction to the study of of the relationships of certain animals. In the tables and figures recording the data obtained in the study of relationships the

homologous reaction is given as 100 per cent. and the heterologous reactions in per cent. of the homologous titer for each antiserum and antigen. The results are given in Tables VIII. to XIII. A part of these results are given in graphical form in Figs. 2

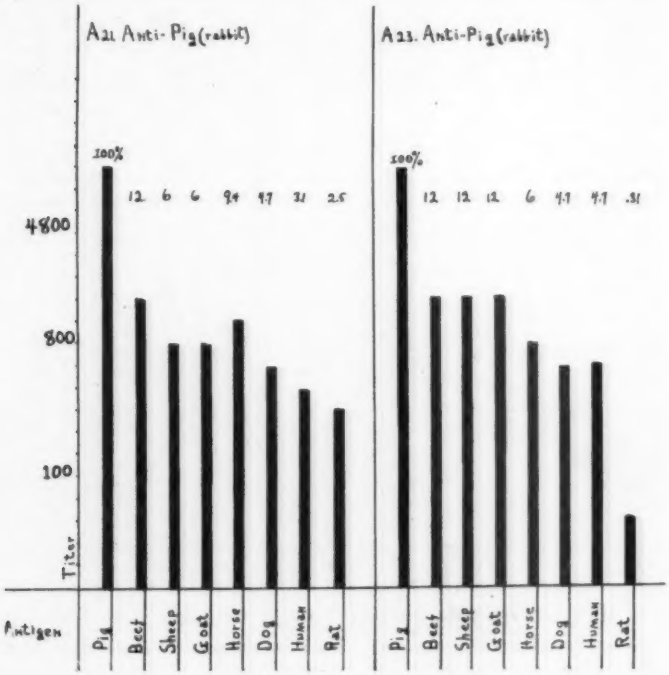


FIG. 2. Comparison of two different anti-pig sera from rabbits. These antisera were of the same homologous titer.

to 8. In these figures the ordinates represent antigen dilution, or titer, given in the geometric progression of the increasing dilutions employed. The homologous reaction is given first in order from left to right along the abscissa and then follow heterologous reactions in the order of relationship to the homologous protein.

Table VIII. gives the reactions of three different antipig sera, two of which were obtained from rabbits and one from a fowl. A21 and C4 were injected with the blood of the same pig, A23 with the blood of another pig. These three antisera were of

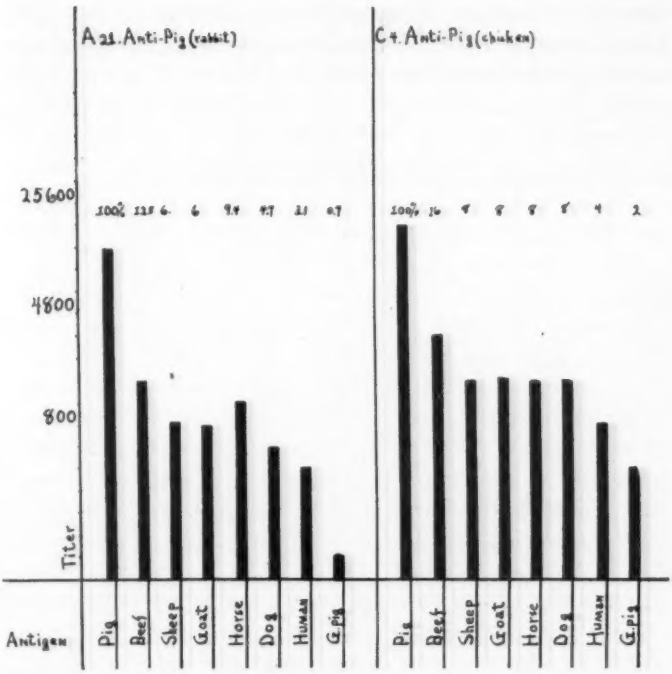


FIG. 3. Comparison of two anti-pig sera of nearly the same strength, one from a rabbit, the other from a fowl.

nearly the same homologous titer. The results are plainly shown in graphical form in Figs. 2 and 3. Considering that the range of error in reading the ring test was 50–100 per cent. there

TABLE VIII.

REACTIONS OF ANTIPIG SERA.

Antigen.	A21 Antipig Rabbit Unfiltered.	A23 Antipig Rabbit Filtered.	C4 Antipig Chicken, Unfiltered 2.25% Saline.
Pig	100 %	100 %	100 %
Beef	12.5	12.5	16.66
Sheep	6.25	12.5	8.33
Goat	6.25	12.5	8.33
Horse	9.4	6.25	8.33
Dog	4.7	4.6	8.33
Human	3.13	4.6	4.16
Rat	2.5	.31	
Guinea pig	.7		2.08
Rabbit			1.04

seems to be essential agreement in the reactions of these sera. This is true even though the antisera were produced in such different animals as rabbits and fowls.

TABLE IX.

REACTIONS OF ANTIBEEF SERA.

Antigen.	A22 Antibeef Rabbit Filtered.	A24 Antibeef Rabbit Before Filtering.	C8 Antibeef Chicken, Filtered .85% Saline.
Beef	100 %	100 %	100 %
Sheep	66	50	100
Goat	50	50	100
Pig	16.66	8.33	12.5
Horse	16.66	8.33	6.25
Dog	16.66	8.33	3.1
Human	8.33	8.33	3.1
Wild rat	.2	.1	1.6

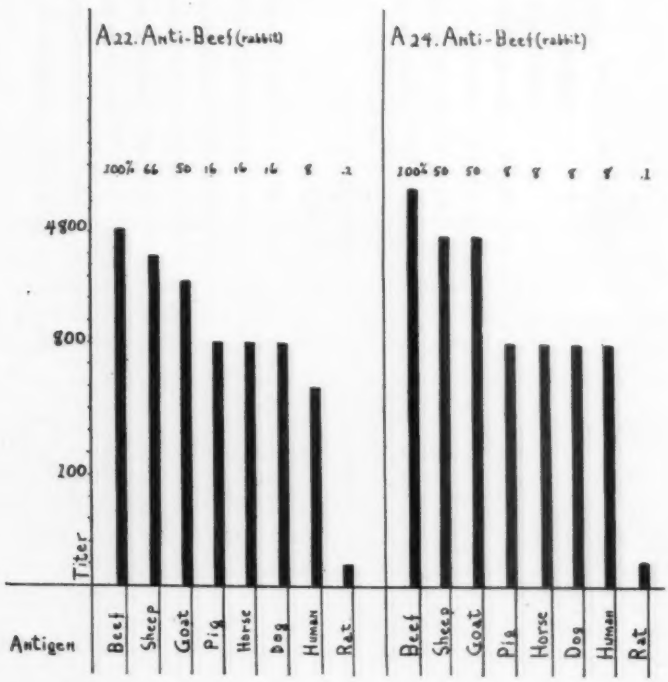


FIG. 4. Comparison of two different anti-beef sera from rabbits.

The animals whose sera are shown are usually considered to be related to each other as follows: pig, beef, sheep and goat belong to the order Artiodactyla; horse belongs to the closely related order Perissodactyla; dog, human and guinea pig belong to the distantly related orders, the Carnivora, Primates and Rodentia respectively. Except for the horse the reactions all correspond to the accepted classification of these animals.

Table IX. and Figs. 4 and 5 show the results obtained with three antibeef sera. Two of these were obtained from rabbits, the third from a fowl. A24 and C8 were injected with blood serum from the same cow. A22 was injected with serum from two different cows. The homologous titers of these antisera were not the same. In spite of this there is again a general correspondence in results obtained with one important exception. The latter is the rodent reaction. Whereas a very low value for rodent blood was given by the rabbit antisera, a very much higher value was obtained in the case of the chicken antiserum. No one has ever been able to produce a rabbit antiserum which would react against rabbit blood. There is therefore an inhibition of precipitin formation in a rabbit when injected with rabbit blood. This same inhibition would be expected to extend to animals closely related to rabbits, that is to other rodents at least. No such inhibition for rodent blood would be expected in chicken antisera and indeed as the data show a much greater reaction is then obtained. It seems therefore that chicken anti-

TABLE X.

REACTIONS OF ANTISHEEP SERA.

Antigen.	P37 Antisheep Rabbit Filtered.	P40 Antisheep Rabbit Filtered.	C11 Antisheep Chicken Filtered, 2.25% Saline.	C17 Antisheep Chicken Filtered, 2.25% Saline.
Sheep.....	100 %	100 %	100 %	100 %
Goat.....	66	75	100	66.66
Beef.....	50	50	75	33.33
Pig.....	8.5	3.1	0.0	.26
Horse.....	4.16	3.1	0.00	1.04
Dog.....	4.16	3.1	12.5	.13
Human...	4.16	.58	0.00	
Tame rat..			0.00	

sera would give us a truer picture of the relationships of rodents to other mammals than would rabbit antisera.

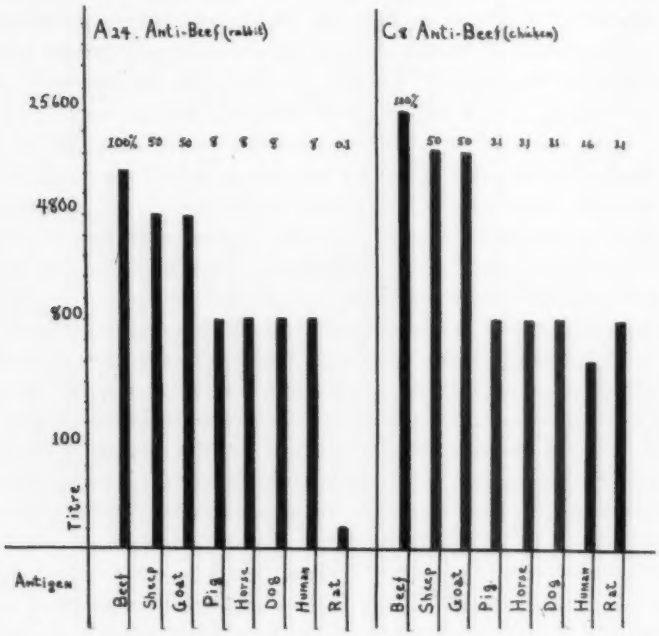


FIG. 5. Comparison of two anti-beef sera, one from a rabbit, the other from a fowl.

Table X. shows the results obtained with four antisheep sera, two obtained from rabbits and two from fowls. Fig. 6 gives a comparison of the two rabbit antisheep sera. While there is a good correspondence in the two sera obtained from rabbits, and this in spite of a great difference in their strength, there is not such a good correspondence in the chicken sera. Some of the values obtained with the chicken sera correspond closely with the others but especially in C11 no reaction for pig, horse, and human blood was obtained at all. Apparently this chicken produced no antibodies for these bloods. This seems evidence for a multiplicity of antibodies present in an antiserum produced in response to the injection of a single blood serum.

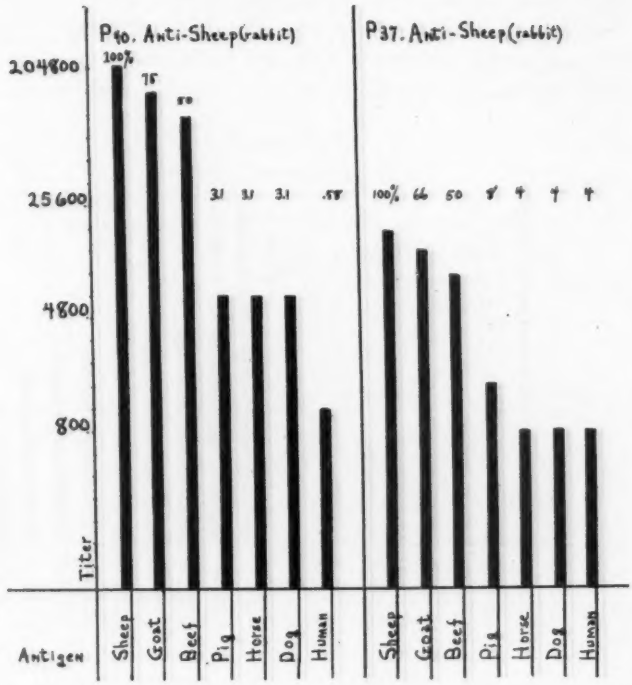


FIG. 6. Comparison of the reactions of two anti-sheep sera from rabbits, the homologous titers being quite different.

TABLE XI.

REACTIONS OF ANTIDOG SERA.

Antigen.	P123 Antidog Rabbit Filtered.	P140 Antidog Rabbit Filtered.
Dog	100 %	100 %
Beef	4.7	12.5
Horse	2.4	12.5
Sheep	2.4	12.5
Goat	1.6	9.4
Human	1.6	9.4
Pig	.8	9.4
Wild rat	0.00	6.25

Table XI. and Fig. 7 give the reactions of two antidog sera obtained from rabbits. It may be seen that the correspondence between these sera is not as close as with the rabbit sera given before. This is true in spite of the fact that both rabbits were injected with the blood from a single dog.

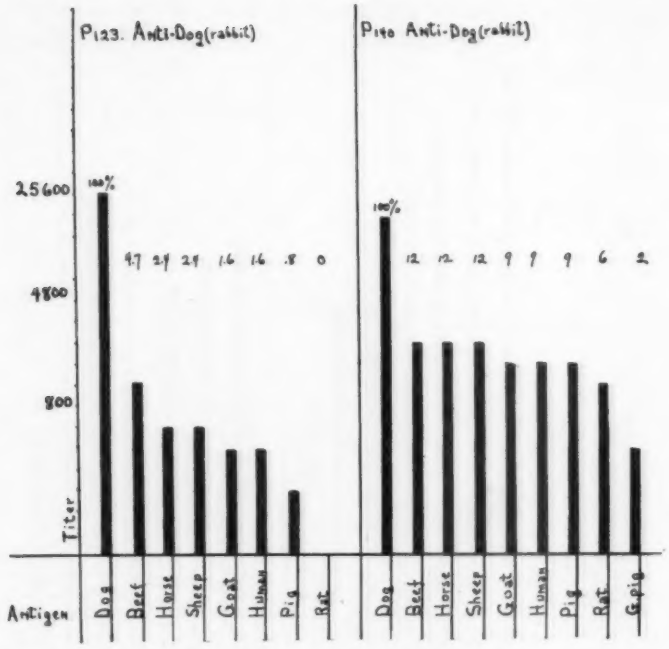


FIG. 7. Reactions of two anti-dog sera from rabbits.

Table XII. shows the reactions obtained with four different antihorse sera, two from rabbits and two from fowls. The blood for these injections was obtained from a single horse. The rabbit sera are shown in Fig. 8. As may readily be seen the poorest correspondence in the whole series of antisera was obtained here.

The data for all these reactions show that there may or may not be correspondence in the reactions of various antisera of the same kind. If this is true how may it ever become reasonably certain that any series of values represents the true relationships of the animals tested? Perhaps there are two criteria by which we may recognize the truth in these reactions. In the first place where a general correspondence occurs in the reactions of two or more antisera of the same kind, produced in similar or different animals, it would seem probable that the reactions represented some reality in the relationships of the animals tested. This would be especially true where an antiserum pro-

duced in a rabbit corresponded closely with one produced in a chicken. In the second place, the relative relationship of two different animals should be the same whichever one of these is used as primary antigen. For instance pig should bear the

TABLE XII.

REACTIONS OF ANTIHORSE SERA.

Antigen.	A25 Antihorse Rabbit Filtered.	P105 Antihorse Rabbit Filtered Twice.	C14 Antihorse Chicken Unfiltered, 2.25% Saline.	C18 Antihorse Filtered, 2.25% Saline.
Horse.....	100 %	100 %	100 %	100 %
Beef.....	6.25	6.25	12.5	3.13
Human...	4.16	1.56		
Goat.....	3.13	0.00		
Dog.....	3.13	1.56	6.25	1.56
Sheep.....	2.0	0.00		.78
Pig.....	2.0	0.00	1.56	1.56

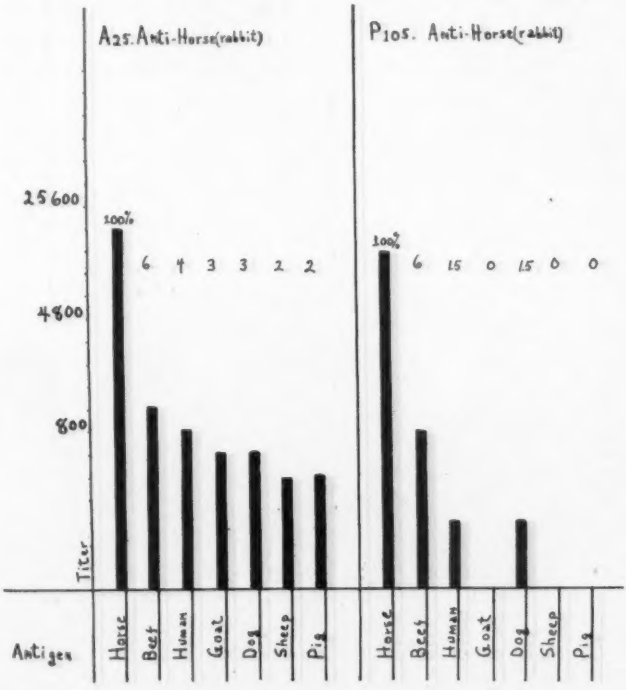


FIG. 8. Comparison of two anti-horse sera from rabbits.

same relationship to beef whether an antibeef serum was titrated with pig or whether an antipig serum was titrated with beef. It would seem then that this principle of reciprocal relationships could be used as a test of the truth of the values obtained in the precipitin reaction. Table XIII. gives the values obtained in these reciprocal titrations. The titrations which failed to exhibit correspondence are marked with an asterisk.

TABLE XIII.

RECIPROCAL VALUES OF ANTISERA.

Antiserum.	Antigen.	Per Cent.	Antiserum.	Antigen.	Per Cent.
Rabbit sera			*Rabbit sera*		
A22 (antibeef).....	Sheep	66	P40 Antisheep	Beef	75
	Pig	16.6	A21 Antipig	"	12.5
	Horse	16.6	A25 Antihorse	"	6.25*
	Dog	16.6	P140 Antihog	"	12.5
A24 (antibeef).....	Sheep	50	P37 Antisheep	"	50
	Pig	8.3	A23 Antipig	"	12.5
	Horse	8.3	P105 Antihorse	"	6.25
	Dog	8.3	P123 Antidog	"	4.7
P37 (antisheep).....	Pig	8.3	A21 Antipig	Sheep	6.25
	Horse	4.16	A25 Antihorse	"	2.08
	Dog	4.16	P125 Antidog	"	2.4
P40 (antisheep).....	Pig	3.1	A23 Antipig	"	12.5 *
	Horse	3.1	P105 Antihorse	"	0.0 *
	Dog	3.1	P140 Antidog	"	12.5 *
A21 (antipig).....	Horse	9.4	A25 Antihorse	Pig	2.08*
	Dog	4.7	P123 Antidog	"	.8 *
A23 (antipig).....	Horse	6.25	P105 Antihorse	"	.00*
	Dog	4.6	P140 Antidog	"	9.4
P123 (antidog).....	Horse	2.4	A25 Antihorse	Dog	3.13
P140 (antidog).....	Horse	12.5	P105 Antihorse	"	1.56*
Chicken sera			*Chicken sera*		
C8 (antibeef).....	Sheep	100	C11 Antisheep	Beef	75
	Pig	12.5	C4 Antipig	"	16.6
	Horse	6.25	C14 Antihorse	"	12.5
C11 (antisheep).....	Pig	0.0	C4 Antipig	Sheep	8.3 *
	Horse	8.3	C18 Antihorse	"	.78*
C4 (antipig).....	Horse	8.3	C14 Antihorse	Pig	1.56*

Twenty reciprocal values have been obtained with rabbit antisera and of these twelve gave correspondence within the

limits of error. Of the eight which failed to give correspondence five involved horse serum and two involved dog serum. Of six chicken reciprocal titrations three gave correspondence and three failed to do so. Two of the latter involved horse serum. Clearly the horse serum was aberrant in its reactions. Nuttall reports similar results with horse serum. There may then be certain animals whose blood sera are not suitable for studies of this kind. If now only the beef, pig, sheep, and dog reciprocal titrations are considered, out of fifteen pairs of values eleven gave agreement, a rather high percentage.

The entire series of reactions recorded in these titrations seems to justify these conclusions:

1. That there is a variation in the response of different animals of the same species to the same protein.

2. That in spite of this variation there is a general agreement in the group reactions obtained with the majority of the antisera tested. This correspondence is independent of the homologous titers of the antisera compared (provided the antisera are sufficiently strong to react with all the proteins tested) and may be found even in antisera produced by such different animals as rabbits and fowls.

3. That in rabbit antisera there is an inhibition of response to other rodent bloods which is absent in fowl antisera.

4. That where there is a variation in the group reactions of two antisera of the same kind it is possible to tell which is the truer series of values by making reciprocal titrations. Only those values which check within the limits of error of the reaction may then be taken.

Discussion.

The building of phylogenetic trees has practically ceased today. This is not by any means because the problems of animal relationships are believed to be finally settled. It seems rather to be an expression of hopelessness as to the possibility of solving these problems. It is true that there is general agreement as to the relationships of animals within certain main branches of the animal kingdom. But there is no such agreement as to the affinities of many of the phyla of Invertebrates to each other or to Vertebrates. But even within these main groups

of animals we have failed to reach agreement as to what constitutes a species or to learn how to express quantitatively the relations of one species or genus or family to another. For classification has been based chiefly on adult morphology, the tendency being to throw aside the evidence from developmental morphology as being more liable to error. However this may be, morphology itself is subject to certain limitations. Chief among these are two (1) an error due to convergence which it is difficult to estimate with certainty, (2) the failure to give a quantitative expression to morphological features in general and hence the necessity of depending much on interpretation as to what various structures may mean in descent. Interpretations differ with interpreters and hence it is that there is endless difference of opinion as to the relationships of certain groups of animals necessitating countless "revisions" of them. The discovery of an objective, quantitative test, which could be applied to animals and through which some general agreement as to their relationships might be attained, would be a great help to the zoölogist.

Attempts to study other than gross morphological structures of animals for the purpose of understanding their relationships have already been made. The study of the distribution of respiratory pigments and related substances is an instance in point. While it has been found that hemoglobin is rather erratically distributed among Invertebrates, the gaps may partly be filled with animals possessing related substances. The study of the crystallography of the hemoglobins by Reichert and Brown (1909) is another instance. The results obtained by these authors confirmed in general the existing classification. Chemical analyses of various tissues have also been made but such methods fail for the most part to show stereochemic differences between substances of essentially the same elementary constitutions. It seems to be true, however, that it is generally such stereochemic differences or resemblances which are most important in the study of related animals. We must have a method therefore which may identify and give some quantitative expression to these differences. Such a method seems available in the precipitin reaction.

Perhaps then the most important contribution to the study of animal relationships, aside from morphology, can be made

through the use of the precipitin reaction. For the results obtained by it can be expressed in a quantitative manner, and are therefore entirely objective and independent of the interpretation of the observer. The use of the precipitin reaction must, however, be carefully controlled. Though this reaction has been shown to be very sensitive, nevertheless it is subject to a fifty to one hundred per cent. possible error as used in these experiments. Furthermore it is incapable of distinguishing between closely related animals. It is affected by the H-ion and salt concentrations, but most important of all *it must be standardized through the use of secondary antigen solutions of known protein content*. With these precautions the results obtained through its use by everyone will become strictly comparable and of considerable value.

From the data on relationships obtained with this limited series of animals it is felt that Nuttall's statement is essentially true. For the degree of reaction between an antiserum and various animal bloods has in general paralleled the relationships among those animals. Thus nearly always the reaction of a given antiserum is greater with animals belonging to the same order than with members of other orders. The only exception to this has been the horse serum (Perissodactyla) which sometimes was reacted upon more strongly or equally strongly by an antipig or an antibeef or an antisheep serum than were some other sera belonging to the order Artiodactyla. The relationship between Artiodactyla and Perissodactyla has always been considered to be close, the two usually being included in one order, the Ungulata. The precipitin reaction reveals this close relationship. The results on horse serum seemed irregular, however, and not too much emphasis is to be put upon them.

When essentially the same series of reactions may be obtained from various antisera of the same kind, whether they be produced in fowls or rabbits, the probability that these reactions correspond to some reality in the relationship of the animals concerned becomes very great. And when, furthermore, the reciprocal values obtained with two or more different antisera check within the range of error, as happened in the majority of these titrations, the probable correctness of this conclusion becomes still greater.

Much criticism of Nuttall's work has appeared in recent years in Europe. Statements have been made that the precipitin reaction did not follow at all the relationships of the proteins tested. The results given here do not support such views. Never has any heterologous reaction in this entire series exceeded the homologous reaction of the same antiserum, as Friedberger and Collier (1919) or Friedberger and Meissner (1923) claim. Perhaps the fault lies with their technique for they have apparently used neither buffered salt solutions nor standard antigens of known strength. Furthermore the dilutions which they made of antigen were only 1–100; 1–200; 1–1,000; 1–10,000; and 1–20,000. The possible error in their readings must therefore be 1,000 per cent. in most cases. If such crude methods had been used in these tests no doubt the results obtained would correspond to theirs but the value of the precipitin reaction in the study of relationships would have been entirely overlooked. A better technique must be used if results of value are to be obtained. It is not that poor results cannot be obtained with this reaction, the important thing is that good results can be obtained and poor results recognized as such and discarded from further consideration.

The fact that the values obtained in this study of a limited series of animals correspond closely with the results of Nuttall's quantitative tests shows that the precipitin reaction can give essential uniformity of results. Having thus proven its value in the study of Vertebrates where relationships are relatively certain, it may now be applied to the study of animals of more uncertain affinities. The Invertebrates offer a large and important field for such research. A few results have already been obtained by Nuttall in the study of Crustacea. Perhaps also his demonstration of the affinity of the King Crab (Limulus) to spiders is to be taken as an indication that the further application of the precipitin reaction to the study of relationships of Invertebrates will eventually yield results of some value to the phylogenist.

Conclusions.

1. The possible error in reading the ring test in this study has been 50–100 per cent.

2. The sensitivity of the ring test is very great.

3. The titer of an antiserum is directly proportional to the concentration of antigen within its limits of reaction.

4. Total nitrogen determinations, at least, should therefore be made on all antigens in order that comparable results may be obtained.

5. The specificity of the reaction of an antiserum decreases with time.

6. The H-ion concentration of antigen solutions affects the reaction. Therefore buffered salt solutions should be used.

7. Increase of salt concentration from .85 per cent. to 2.25 per cent. decreases the titer of chicken antisera and tends to increase their specificity.

8. Filtration of antisera through Berkfeld filters usually does not decrease titer nor change specificity.

9. There is a variation in response of different animals of the same species to the same protein.

10. There was a general agreement in the group reactions obtained in the majority of cases. This correspondence was independent of the strength of the antisera used and occurred in antisera produced in such different animals as rabbits and fowls.

11. There is an inhibition of the response of rabbit antisera to other rodent bloods which is absent in the fowl.

12. The principle of reciprocal relationships can be used to test the agreement of the values obtained.

13. The ring test is quantitatively specific, for no heterologous reaction ever exceeded the homologous reactions of the same antiserum.

14. The ring test when properly performed may give information of value to the student of animal relationships.

The writer wishes to express his appreciation for the aid and criticism of his work on the part of Dr. M. F. Guyer, Dr. A. S. Pearse, Dr. H. C. Bradley and Dr. W. J. Meek of this University.

LITERATURE.

Ascoli, M.

'02 Ueber den Mechanismus der Albuminurie durch Eiereiweis. Munchen med. Wochenschr., Jahrg. XLIX., pp. 398–401.

Bordet, J.

'99 Sur l'agglutination et la dissolution des globules rouges par le serum d'animaux injectes de sang defibrine. Ann. de l'Inst. Pasteur, XIII., pp. 688–695.

Clark, W. M.

'22 The Determination of Hydrogen Ions. 2d ed. Williams & Wilkins, Baltimore.

Dervieux.

'21 Procede de diagnostic individuel du sang et de sperme. Compt. rend. d'Acad. d. Sc., Vol. 172, p. 1384.

Evans, A. C.

'22 A Buffered Physiologic Salt Solution. Journ. Infec. Dis., Vol. 30, 1922, p. 95.

Folin, O., and Wright, L. E.

'19 A Simplified Macro-Kjeldahl Method for Urine. Journ. Biol. Chem., Vol. 38, p. 461.

Fornet, W., and Muller, M.

'08–'09 Zur Herstellung und Verwendung prazipitierender Sera, insbesondere für den Nachweis von Pferdfleisch. Zeit. für Biol. Technik und Methodik, Vol. 1, p. 201.

'10 Praktische und Theoretische Prazipitin Untersuch. Zeit. für Hygiene, Vol. 65–66, p. 215.

Friedberger, E., and Collier, A.

'19 Ueber heterogenetische Antigene und Antikorper VII Mitteilung. Heterogenetische Prazipitine. Zeit. für Immunitatsforschung, Bd. 28, I., Orig., p. 237.

Friedberger, E., and Jarre, H.

'20 Ueber Aspecifische prazipitinierende Sera. Zeit. für Immunitatsforschung, I., Orig., Bd. 30, p. 351.

Friedberger, E., and Meissner, G.

'24 Typus und Wesen der isogenetischen verwandtschafts und heterogenetischen Prazipitation mit monagenen Seris. Zeit. für Immunitatsforschung, Bd. 36, I., Orig., p. 283.

Geyer, K.

'13 Untersuchungen über die chemische Zusammensetzung der Insekten hamolymph und ihre Bedeutung für die Geschlachtliche Differenzierung. Zeit. für Wissenschaft. Zool., Vol. 105, p. 348.

Guyer, M. F.

'25 Soma and Germ. Amer. Nat., Vol. LIX., p. 97.

Guyer, M. F., and Smith, E. A.

'20 Studies on Cytolysins. II. Transmission of Induced Eye Defects. Journ. Exper. Zoöl., Vol. 31, p. 171.

Hektoen, L.

'18 Production of Precipitins by the Fowl. Journ. Infec. Dis., Vol. 22, p. 561.

'22 Precipitin Reactions of the Normal and Cataracterous Lens. Journ. Infec. Dis., Vol. 31, p. 72.

Hektoen, L., and Manly, L. S.

'23 Specific Precipitin Reaction of Semen. Journ. Infec. Dis., Vol. 32, p. 167.

Hektoen, L., and Schulhof, K.

'23 On Specific Erythroprecipitins (Hemoglobin precipitins?). II. Hemoglobin precipitins in Identification of Blood. Journ. Infec. Dis., Vol. 33, No. 3, p. 224.

'24 Further Observations on Lens Precipitins. Antigenic Properties of Alpha and Beta Crystallins. Journ. Infec. Dis., Vol. 34, p. 433.

Hektoen, L., and Welker, H.

'24 Precipitin Reactions of Serum Proteins. Journ. Infec. Dis., Vol. 35, p. 295.

Kister, J., and Wolff, H.

'02 Zur Anwendbarkeit der serodiagnostischen Blutprufungsverfahrens. Zeitschr. F. Hygiene, Bd. XLI., p. 410.

Kraus, R.

'97 Ueber specifische Reactionen in keimfrein Filtraten aus Cholera, Typhus, und Pestbacillen Culturen, erzeugt durch homologes Serum. Wien. Klin. Wochen., Vol. 32, p. 736.

Mason, V. R.

'22 The Relation of the Hydrogen-ion to Specific Precipitation. Johns Hopkins Hosp. Bull., Vol. 33, p. 116.

Mason, E. C., and Sanford, A. H.

'24 A Proposed Buffered Physiologic Salt Solution to be used in Serology. J. Lab. and Clin. Med., Vol. IX., No. 5.

Nuttall, G. H. F.

'02 Progress Report upon the Biological Test for Blood as applied to over 500 Bloods from various sources, together with a preliminary report upon a method of measuring the degree of reaction. Brit. Med. Journ., Vol. I., p. 825.

'04 Blood Immunity and Blood Relationship. Cambridge Univ. Press, 1904.

Reeser, H. C.

'19 Mededeelingen von de Ryksserumrichting, Deel. II., Aff. II., Rotterdam.

Reichert, E. T., and Brown, A. P.

'09 The Differentiation and Specificity of Corresponding Proteins and other Vital Substances in Relation to Biological Classification and Organic Evolution. The Crystallography of Hemoglobins. Pub. No. 116, Carnegie Institution of Wash.

Strube, G.

'02 Beitrage zum Nachweis von Blut und Eiweiss auf biologischem Wege. Deutsche med. Wochen., Jahrg. XXVIII., p. 425.

Tchistovitch, Th.

'99 Etudes sur immunisation contre l'anguilles. Ann. de l'Inst. Pasteur, Vol. XIII., p. 406.

Uhlenhuth, P.

'01 Weitere Mittheilungen über meine Methode zum Nachweise von Menschenblut. Deutsche med. Wochen., Jahrg. XXVII., p. 260.

Yu, I.

'23 Untersuchungen über heterologous Prazipitation. Central. für Bakter., Parasitenkunde und Infectionskrankheiten, Vol. 90, Orig., p. 381.

NOTES ON THE FORM AND FUNCTION OF THE GOLGI APPARATUS IN STRIATED MUSCLE.

ROBERT H. BOWEN,

DEPARTMENT OF ZOÖLOGY, COLUMBIA UNIVERSITY.

It is a curious, not to say remarkable, fact that among the hundreds of papers published since 1898 on the morphology and function of the Golgi apparatus and the mitochondria, very few indeed have been seriously concerned with these cytoplasmic elements as they presumably occur in striated muscle "cells." This undoubtedly means, not that the matter has been overlooked, but that it has been looked over and found very difficult of solution. The whole question of the structure of striated muscle being still in a somewhat uncertain state, it is not surprising that the morphology of its generalized cytoplasmic components should likewise be unsettled. Indeed this could be anticipated, since the extraordinary differentiation of muscle fibers may well involve morphological modifications of the Golgi apparatus and mitochondria of such a nature as to render uncertain their immediate identification. Still it is clear that an understanding of the morphological disposition of these cytoplasmic elements might throw much interesting light not only upon their own mode of functioning but upon that of muscle substance as well.

The tendency in recent years, upon such infrequent occasions as the matter has been mentioned, seems to have favored the identification of the so-called Cajal-Fusari network [1] with the customary Golgi apparatus of other tissues. The morphology of this network, first described by Cajal and later by Fusari, was subsequently studied in great detail by Veratti ('02),[2] whose

[1] It should be carefully noted that the terms "network" and particularly "reticular apparatus" have been frequently used in discussing this matter in striated muscle without any intention of implying that the material thus denominated is necessarily homologous with the true Golgi apparatus or network.

[2] This paper contains a very complete bibliographical review of the whole subject, to which reference may be made for the papers of Cajal and Fusari. These were unfortunately published for the most part in journals not readily accessible to

paper is illustrated by a remarkable series of figures. Veratti found, in essential agreement with Cajal and Fusari, that there is present in the striated muscle fiber an elaborate system of very delicate fibers, which form an anastomosing network rather easily blackened in a specific manner by the so-called black reaction (reazione nera) of Golgi. This network consists primarily of a series of regularly arranged cross-fibers, located in various but definite planes as determined by the phenomena of cross-striation in the muscle fiber itself. These cross-threads are connected by more irregularly arranged fibers running parallel to the muscle columns. This whole network has been shown with reasonable certainty to occupy the interstices between the muscle fibrils, lying thus in the sarcoplasm but having an entirely independent existence. The general accuracy of these findings has subsequently been substantiated by Martinotti ('04), Sánchez ('07) and particularly Holmgren ('08).

It was shown, furthermore, especially by Veratti that the regular arrangement of the network is gradually arrived at by progressive differentiation of a network which is much less regularly arranged in the embryo, for example of mammals. Martinotti ('04) was able also to show that a return to a more or less embryonic condition of the network could be effected in the rabbit by starvation, or section of the fibers of the nerve supply. But in this direction Veratti went still further by demonstrating that the condition of the network at birth varies in different mammals. Thus in the mouse and rabbit, the final arrangement of the net is not achieved until some days after birth; while in the guinea-pig, the final (adult) condition is present at birth, and an arrangement comparable to that of the mouse at term, can be found only in the guinea-pig fetus about fifteen days before birth. In this connection also it is of interest to note that the results for instance of M. Sánchez ('16) on the Purkinje cells of rabbits, show that the Golgi apparatus in these cells only attains its adult development and arrangement by a gradual

most American workers, but a general idea of the results can be found in Cajal ('90) and Fusari ('94). It may also be noted here that the relation of the Cajal-Fusari network revealed by silver nitrate, to the network in striated muscle stained by gold chloride, is still a matter of doubt. In these notes, the latter network will not be considered.

evolution in the first weeks after birth. This at least suggests the possibility that in other nerve cells the adult arrangement of the Golgi material may likewise be a product of post-embryonic development—depending of course in detail upon the kind of cell and its time of functioning with respect to birth, etc.

I have digressed here into these details of the differentiation of the Golgi network because of their remarkable connection with the relations recently developed by Tilney, between the beginning of functional activity of a given muscle and the time of myelinization of the corresponding nerve tracts. It would appear that the nerve fiber is not the only part of the mechanism which at birth is still in an incomplete state, but that the muscle fiber and possibly also the nerve cell itself have still to perfect their organization subsequent to birth. Here the agreement in the guinea-pig between the completion of myelinization before birth as noted by Tilney and the perfection of the muscle network as noted by Veratti, with the known activity of the newborn young of this animal, seems to me of extraordinary interest. It thus appears that the failure of the nerve-muscle mechanism to operate immediately after birth may be due not only to the lack of the myelin sheaths but to the fact that the system as a whole is still in process of completion.

The possible relation of this muscle reticulum to the Golgi apparatus was first discussed by Veratti, who refrained from drawing any definite conclusion. Sánchez ('07), however, concluded that this network represents the apparatus of Golgi-Holmgren, and though he had in mind Holmgren's conception of a trophospongium, it is equally clear that he intended an homology with the Golgi apparatus as conceived by other workers. At about the same time Holmgren ('08) published an account of the network in many kinds of muscle cells, and endeavored to prove that it represented the trophospongium described by him in other kinds of tissue. Golgi himself seems to have accepted the homology of the muscle reticulum with his 'internal reticular apparatus.' In his review of 1911, Duesberg considered the case unproved, but in 1914 he states that "the hypothesis of Golgi is probably correct."[1] The only obvious criticism of this

[1] The not very convincing account and figures of Luna ('11) on mammalian heart muscle must thus be considered as probably incorrect.

conclusion which occurs to me is that in insects the Golgi apparatus seems characteristically to occur in the form of scattered Golgi bodies, rather than a network comparable to the condition usually found in mammals. On the other hand, the silver methods have been tried so often, and by so many people, that if there is a Golgi apparatus in the insect muscle fiber other than the network mentioned, it ought by every possibility to have come to light. It may therefore be tentatively concluded that the so-called muscle "network of Cajal-Fusari" represents the Golgi apparatus (and all of it) in striated (skeletal and heart) muscle fibers of both vertebrates and invertebrates.

It is to be noted that all the results thus far obtained have depended upon the use of silver nitrate, usually following the Golgi fixation in osmium-bichromate. It would be interesting to know whether other methods, which have proved specific for the Golgi apparatus, would reveal the same network, or material arranged in some new way. Recently while studying some preparations of Cowper's gland from the cat, I found some fragments of striated muscle which, inadvertently included with the glandular tissue, had been impregnated by osmic acid according to Nassonov's ('24) modification of Kolatchef's method. Following this treatment a great variety of pictures results. The cross striations may be very clearly demonstrated, after the well-known reaction of osmic acid on striated muscle. Various other ways of impregnating the muscle substance also occur, of which an interesting one is the specific blackening of the sarcoplasm while the muscle columns or fibrils are relatively unstained. A cross-section of a portion of a muscle fiber thus impregnated is shown in Fig. 1.

In one case a very unusual impregnation was found, in which both muscle fibrils proper and sarcoplasm failed to blacken in the least, while fragments of a network lying between the muscle columns were rather clearly impregnated. A small section of such a muscle fiber is shown in Fig. 2. The muscle columns are indistinctly differentiated as longitudinal bands. The network consists of longitudinal and transverse threads along which granular thickenings occur at rather regular intervals. Except for the fact that the longitudinal fibers are unusually numerous

(but see Martinotti '04), these threads seem to correspond in a general way to the Cajal-Fusari reticulum, here revealed in a fragmentary manner.

Added proof is thus brought that this network, responding to the silver nitrate and osmic methods for impregnating Golgi material, actually is the homologue of the Golgi apparatus. It

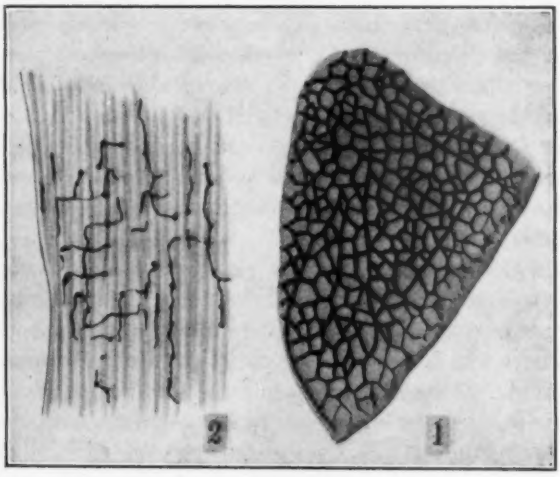


FIG. 1. Portion of a cross-section of a striated muscle fiber from the cat, showing the sarcoplasm impregnated with osmic acid; sarcolemma appears along the right-hand border. FIG. 2. Longitudinal section of the same kind of muscle, showing fragmentary impregnation by osmic acid of the muscle reticulum of Cajal-Fusari (= Golgi apparatus?). Both figures × 2150.

seems to me that the final demonstration of this homology can be obtained only by a study of the development of the network from a stage in which the original condition of the Golgi apparatus will permit its identification beyond dispute. Such an identification seems to have been made by Fañanás ('12), who claims that in the chick embryo (from the sixth day on) the Golgi apparatus of the muscle cells (tubular phase) can be made out as granules and rods localized in the protoplasmic axis of the fiber. Concerning the forms and metamorphosis undergone by this material until the complicated net of the mature muscle fiber is completed, he states that he will treat in detail in a special paper. But I can not find that this paper has appeared.

Assuming that the Cajal-Fusari reticulum actually is the Golgi apparatus of a striated muscle fiber, there is raised at once the interesting question of the functional relation of this material to the functional activity of the muscle itself. It was thought by Cajal that the network, in the insect muscle upon which he worked, was in one sense or another a continuation of the tracheal network. This view was supported by Sánchez ('07), and elaborately developed by Holmgren ('08), who found in the network the trophospongium of his familiar hypothesis. According to Holmgren, in insects this network is in reality a protoplasmic extension of tracheal end cells located on the surface of the muscle fibers; while in vertebrates (and other forms where tracheal tubes are lacking) it is a similar extension of materials essentially to be related to the sarcolemma, or "trophocytes" connected with it. To most workers this would probably seem a very perilous expedient for saving the homologous nature of these networks, especially in view of the fact that the trophospongium in many kinds of cells has been shown to be the Golgi apparatus pure and simple, and quite lacking in its supposed relationship to external trophic cells. As a matter of fact, the supposed relation of the network to the tracheal tubes in insect muscle fibers was investigated and discussed by Veratti, who denied the connection described by Cajal (and later Holmgren). To me the evidence seems to indicate that the networks are essentially independent of extra-muscular formations, but that possibly in insects, tracheal endings may be closely associated with the fibers of the network in a purely spatial sense. While therefore rejecting Holmgren's theory, it is nevertheless interesting to note further that he found in this network an area of metabolic transformations comparable to that of the trophospongium in other cells.

Bearing in mind these facts of morphology and supposed functional significance, it has proved interesting to me to consider the problem in the light of the recent development of opinion on the rôle of the Golgi apparatus in cells generally. From this it begins to appear that the Golgi apparatus is very generally involved in the synthetic operations leading to the formation of various secretory products, including many extra-

cellular enzymes. When, therefore, we find an apparent homologue of the Golgi apparatus in such extraordinary relations to the contractile material of muscle fibers, the suggestion comes to us at once that the functional significance of the Golgi apparatus may here be of the same general character as in at least some other kinds of cells. Thus we might look upon the Golgi apparatus in muscle cells as the possible seat of oxidase formation. Such possibilities are not, however, within the range of cytological examination at present, and some more tangible suggestion will be essential if the matter is to be carried further.

It happens that a very large mass of evidence has already been accumulated, especially through the work of Holmgren ('08, '10, '13, etc.), concerning the positional relationship of the 'Golgi apparatus' in muscle fibers to the so-called Q- and J-granules (or sarcosomes), as well as the remarkable behavior of these bodies during the cycle of muscle action. It has been demonstrated, particularly by Holmgren ('10), that these granules undergo most remarkable changes in staining capacity coincident with the contraction and relaxation wave. These changes, furthermore, are of such a character as to indicate that they are probably related to a transfer of material between granules and muscle columns, presumably necessary to muscular activity. In addition, Holmgren ('10 and '13), Knoche ('09) and others, following up the discoveries of earlier workers, have shown that these granules present many remarkable similarities in staining behavior to the so-called half-moon bodies long since described by Heidenhain in the pelvic gland cells of salamanders. There is here the distinct suggestion that the sarcosomes (*i.e.*, the Q- and J-granules) are actually a specialized type of secretory granule, related primarily to the Golgi network of the muscle fiber, and undergoing a well determined cycle of exhaustion and replenishing synchronous with the contraction and rest of the adjacent muscle columns.

It is not my intention to enter here into a detailed development of these speculations, for which Holmgren's papers suggest many interesting possibilities. Such speculations are hardly warranted as yet, especially because of the conflicting data concerning the distribution of the mitochondria in striated

muscle fibers. This matter has in fact received no very extensive examination, but several workers, and their opinion is concurred in by Duesberg ('10 and '11), have identified the Q- and J-granules as the mitochondrial constituents of striated muscle. But the staining behavior of these bodies changes rapidly in a manner not characteristic of chondriosomes and the evidence that they are of such a nature seems to depend on what might be termed an argument from logical necessity, viz., these bodies sometimes stain like chondriosomes and they have some appearance of being the only granules available which could be so identified. As a matter of fact, a figure of heart muscle given by the Lewises ('24, Plate VI., Fig. b), and Duesberg's ('10) Fig. 24 of the voluntary muscle of the fowl, seem to indicate that the mitochondria are really not the Q- or J-granules at all, but granules with a quite different disposition. The matter evidently is one to be analyzed further, using some basis of identification other than that of staining reactions.

In conclusion, therefore, my purpose in this short résumé has been to bring out our uncertainty concerning some very essential structures in striated muscle fibers; at the same time to indicate the probable disposition of the Golgi apparatus, and to point out various possibilities which this disposition at once suggests. We have now to reëxamine the whole problem in order to establish the homology of the network with the Golgi material of embryonic cells; and to determine the true significance of the Q- and J-granules—whether chondriosomes, possible secretory granules or some as yet unrecognized combination of the two. The solution of these problems should throw much interesting light on the functional significance of the Golgi apparatus, and perhaps also of the mitochondria.

LITERATURE CITED.

Cajal, R. y.

'90 Coloration par la méthode de Golgi des terminaisons des trachées et des nerfs dans les muscles des insectes. Zeitsch. f. wiss. Mikr., Vol. 7.

Duesberg, J.

'10 Les chondriosomes des cellules embryonnaires du poulet et leur rôle dans le génèse des myofibrilles. Arch. f. Zellforsch., Vol. 4.

'11 Plastosomen, "Apparato reticolare interno," und Chromidialapparat. Anat. Hefte, 2. Abt., Vol. 20.

'14 Trophospongien und Golgischer Binnenapparat. Anat. Anz., Ergänz. Heft to Vol. 46.

Fañanás, R. y.

'12 Nota preventiva sobre el aparato reticular de Golgi en el embrión de pollo. Trab. del Lab. de Inves. biol. de la Univ. Madrid, Vol. 10.

Fusari, R.

'94 Étude sur le structure des fibres musculaires striées. Arch. ital. de Biol., Vol. 22.

Holmgren, E.

'08 Ueber die Trophospongien der quergestreiften Muskelfasern, nebst Bemerkungen ueber den allgemeinen Bau dieser Fasern. Arch. f. mik. Anat., Vol. 71.

'10 Untersuchungen ueber die morphologisch nachweisbaren stofflichen Umsetzungen der quergestreiften Muskelfasern. Arch. f. mik. Anat., Vol. 75.

'13 Von den Q und J Koernern der quergestreiften Muskelfasern. Anat. Anz., Vol. 44.

Knoche, V.

'09 Ueber die Struktur der sogen. "interstitiellen Koerner" (Koelliker) der Fluegelmuskelfasern der Insekten. Anat. Anz., Vol. 34.

Lewis, W. H., and Lewis, M. R.

'24 Behavior of cells in tissue cultures. Section VII in General Cytology, edited by E. V. Cowdry, Chicago.

Luna, E.

'11 Sulla fine struttura della fibra muscolare cardiaca. Arch. f. Zellforsch., Vol. 6.

Martinotti, C.

'04 Contributo allo studio dell'apparato reticolare nei muscoli striati di alcuni mammiferi. Giorn. della R. Accad. di Med. di Torino, Vol. 67.

Nassonov, D.

'24 Das Golgische Binnennetz und seine Beziehungen zu der Sekretion. (Fortsetzung). Arch. f. mik. Anat. und Entwick., Vol. 100.

Sánchez, D.

'07 L'appareil reticulaire de Cajal-Fusari des muscles striés. Trav. du Lab. de Rech. biol. de l'Univ. Madrid, Vol. 5.

Sánchez, M.

'16 Recherches sur le réseau endocellulaire de Golgi dans les cellules de l'écorce du cervelet. Trab. del Lab. de Inv. biol. de la Univ. Madrid, Vol. 14.

Veratti, E.

'02 Richerche sulla fine struttura della fibra muscolare striata. Mem. del reale Ist. Lombardo di Sci. e Lett., Vol. 19.

THE SPERMATOGENESIS OF *UMBRA LIMI* WITH SPECIAL REFERENCE TO THE BEHAVIOR OF THE SPERMATOGONIAL CHROMOSOMES AND THE FIRST MATURATION DIVISION.

JAMES O. FOLEY,

ZOÖLOGICAL LABORATORY, UNIVERSITY OF WISCONSIN.

INTRODUCTION.

Recent advances in the genetics of the Teleosts have given an impetus to the study of the chromosomes of fishes. The discovery of an XX, XY type of sex-linked inheritance in *Lebistes reticulatus* (Schmidt, '20; Winge, '22), *Aplocheilus latipes* (Aida, '21) and *Platypœcilus* (species?) as studied by the writer,[1] indicating an XX, XO type of sex-linked inheritance, all point to the presence of heterochromosomes in the fishes, although none have as yet been recorded. Winge ('22) suggests the possibility of such elements existing in *Lebistes* but gives no cytological evidence. Geiser ('24) says in discussing the subject in *Gambusia*, "If one were to trust to the uncertain help of analogy, it might be claimed that *Gambusia* has one heterochromosome." Agar ('11) shows in the heterotypic division of *Lepidosiren paradoxa* (although he does not indicate them as such), two large chromosomes, almost identical as to size and shape. These chromosomes show a tendency to behave like the sex-elements in those forms in which they have been definitely worked out. The nature of these chromosomes and their similarity of size and shape would seem to indicate, if they are sex-chromosomes, that in *Lepidosiren* the male is homozygous for sex and should show the poultry type of sex-linked inheritance.

Upon reviewing the literature on the spermatogenesis of the Teleosts, it becomes apparent that there are many features in the germinal cycle of these forms which are not clear, and that these should be studied further before proceeding with the problem of the sex-chromosomes. During the summer of 1923 a

[1] Data as yet unpublished.

great number of different kinds of fish were collected (bass, perch, blue-gills, sunfish, stickleback, pickerel, carp, and mud-minnows) and their testes preserved, sectioned, and examined. After careful examination the mudminnow (*Umbra limi*) was selected as the most favorable specimen for study. *Umbra* offers especially good material as the spermatogonial cells are large, with the chromosomes in the form of L's and V's. They also show no great tendency to clump, hence individual chromosomes and chromosome pairs may be picked out with a fair degree of certainty. The first maturation division is especially clear, showing definite meiotic stages and clean separation of the tetravalent chromosomes at the metaphase so that, in the selection of this form, the writer believes that he has avoided the main difficulties encountered by most investigators on fish spermatogenesis, such as: (1) The small size of the germ-cells, especially the primary and secondary spermatocytes; (2) the small size of the chromosomes together with the fact that they usually are in the form of threads, rods, or dots, rendering counting extremely difficult and making it practically impossible to follow them through the various stages in spermatogenesis; and (3) the tendency of the chromosomes to clump badly.

The purpose of the present paper is to try to clear up some of the little known or little understood phases in the spermatogenesis of the bony fishes. The writer wishes to express his appreciation of the aid of Dr. A. S. Pearse in collecting specimens and in reading the manuscript, of Dr. L. J. Cole for pointing out the genetic application of the problem, and of Dr. M. F. Guyer for suggestions in technique and helpful criticisms on the entire work.

Materials and Methods.

The fishes used in this investigation were collected in the vicinity of Madison, from Lake Mendota and from various small streams which empty into Lake Mendota and Lake Wingra. Collections of *Umbra limi* were made once a week and testicular material was fixed every day, or every other day during the time the cells in the testes were undergoing proliferation. The testes are two elongated white bodies situated in the posterior part of the body-cavity just ventral to the swim-bladder. They

are oval in shape tapering from the middle toward each end. The testes do not fuse as they do in many fish but remain separate throughout their entire length. It was found that material from fish which were kept in the laboratory for more than a week could not be relied upon, as it began to show abnormal behavior.

For cytological study material was fixed in Carnoy's, Gilson's and Flemming's strong fluids, Flemming's modified according to Hance ('17), and Bouin's fluid modified according to Allen ('16). Allen's modification of Bouin's fluid was found to give the best results, although Hance's modified Flemming gave fair fixation. The testes were dissected out and quickly immersed in the fixative, and then cut into very small pieces. The fixative was kept at the same temperature as the water of the aquarium which contained the fish. Safranin and gentian violet, or safranin counterstained with licht-grün or iron-alum hæmatoxylin counterstained with eosin, acid fuchsin, picric acid, licht grün, or Congo red were employed as stains. The best results with counterstains following Bouin's fixation were secured with solutions of licht grün and Congo red in 95 per cent. alcohol. Sections were cut from three to seven micra in thickness. Those of seven micra were best for studying the chromosomes and for making chromosomal counts in the large spermatogonial cells. Smears were also made but did not prove as valuable as the sections in this study.

Seasonal Cycle in the Spermary of *Umbra*.

The testes of *Umbra* are usually full of spermatozoa up to the middle of May. Though there is some variation among individuals and in different years on account of differences in seasonal succession, expulsion of the spermatozoa usually occurs during the last week or ten days of May. Following this there is a period of rest in the testes until about the middle of July. The primordial germ cells or primary spermatogonia, as I shall designate them in this paper, are arranged around the periphery of the lobule before and just following the expulsion of the spermatozoa. This peripheral position of the primary spermatogonia in those lobules in which a few spermatozoa remain,

give such lobules a superficial resemblance to the seminiferous tubules of the higher vertebrates. Turner ('19) describes a similar method of peripheral arrangement in the pickerel. There is very little if any division of the primary spermatogonia during this period in the testes. Each new spermatogonium is formed by a process of growth from a migrating cell; once established, it eventually becomes the center of a cyst within the lobule. This process of cyst or pocket formation begins at the periphery and gradually extends toward the center of the lobule until the latter is filled with a solid cord of germ-cells. Following the formation of this primary cord of cells, other small migrating cells may be seen along the periphery of the lobule or between the cyst walls during the period of migration and growth which reaches its apex by the middle of July. Up to this time few mitotic figures can be seen. From the middle to the last of July there is a period of rapid multiplication of the primary spermatogonia and in a number of the cysts the cells have advanced as far as the first maturation division. By the first week in August the cells in a number of cysts have completed maturation and show various stages in the transformation of the spermatids. During the remainder of August and early September the various maturation stages are to be found in abundance. By the middle of September a large proportion of the testes are filled with spermatozoa. With the completion of the transformation of the spermatids into spermatozoa all evidence of cyst arrangement within the lobule disappears. Just before and immediately after expulsion of the spermatozoa, which occurs during the following season, there is no evidence of cysts within the lobules. They are formed again at the beginning of the next migration and multiplication period.

There is no antero-posterior seriation in the testis of *Umbra;* each cyst completes its cycle as an independent unit. A cyst in which late primary spermatocyte figures can be seen, however, may also show earlier maturation divisions and some spermatogonia. Likewise secondary spermatocytes and transforming spermatids may be found in the same cyst. This makes detection of the different stages in the spermatogenetic cycle more difficult. Another factor which complicates the study is the

seasonal variation which influences, to some extent, the seasonal cycle in the testis. During the summer of 1924, a comparatively wet and cold season, testicular cysts which during a normal season would show a great many maturation divisions had not advanced any further than the formation of spermatogonia.

Spermatogonia.

1. *Origin.*—How is the new crop of germ-cells formed at the beginning of each seasonal cycle? Are they produced from reserve germ-cells which in turn are descended from other reserve cells, or are they formed anew from migrating cells which come from some point outside the lobule? Turner ('19), found the latter to be true for the perch. Hargitt ('24), working on the adult salamander (*Diemyctylus viridescens*), found that the spermatogonia are formed anew each season from the epithelial cells which line the collecting tubules or from the peritoneal tissue which covers the testis. Although no effort has been made by the writer to trace the origin of the early spermatogonia outside the testis, there seems to be little doubt that in *Umbra*, as in the perch, the primary germ-cells or early spermatogonia arise anew each season from cells which migrate into the lobules from some outside point. A certain amount of migration and growth, indeed, seems to be in progress throughout the entire cycle of spermatogenesis. Occasional small ameboid cells are to be seen along the lobule wall or between the walls in small numbers during August and early September when the testes are filled with spermatogonia, spermatocytes or spermatids and spermatozoa. In material collected from the first to the middle of September, during which time most of the testis becomes filled with spermatozoa, single large resting cells or clusters of cells may be found scattered throughout the lobules. Whether these degenerate or remain in a resting state with an occasional division is not known. Clusters of large cells are found in isolated cases still persisting in the fall but since no such cysts are found the following spring, it is inferred that they have given rise to spermatozoa during the winter. The large isolated cells which may be found along with a few ameboid cells in lobules which have completed their cycle of spermatogenesis may

remain over as suggested, or they may degenerate. No positive evidence can be presented on this point although similar large cells, showing occasional mitosis, can be observed throughout the time that the testes are filled with spermatozoa.

The origin of the early spermatogonia can be best observed just before the liberation of the spermatozoa. At this time the new germ-cells, which are to be found around the periphery of the lobule, are of varying sizes, ranging from small migrating cells (Fig. 1) to very large cells (Fig. 2) in a growing or resting condition. Of twenty-four linear measurements taken at random, cells were found to range from 19.50 to 58.50 micra. The migrating cells (Fig. 1) are ameboid in shape and have a darkly staining nucleus with a thin investing sheath of cytoplasm closely applied to the nuclear membrane. The nuclei take the stains more deeply than the nuclei of the large resting cells in the same lobule. The migrating cells (Fig. 1) are oblong or flattened with a prominent chromatin nucleolus suspended in the nuclear reticulum near the nuclear membrane. The chromatin granules are quite large and peripherally arranged around the nuclear membrane.

2. *Growth of the Spermatogonia.*—As soon as the migrating germ cells become lodged at the periphery of the lobule the growth period of the spermatogonia begins. This growth period is of special interest in *Umbra* and other Teleosts for in these forms the great increase in cell size during spermatogenesis is thrown back into the early spermatogonial stages. In most animals the growth period occurs in the primary spermatocyte stage and it is usual to find the spermatogonia of almost equal size. The precocious growth period of Teleosts may be due to a number of factors: (1) All the spermatogonia formed during any one season are ultimately used up. (2) The method of cyst-formation in the lobule, in which each migrated germ-cell becomes the primary center of a cyst; since each seasonal crop of germ-cells arises anew from migrating cells. (3) The long period of rest in the testis following its depletion of spermatozoa, during which time there is an active reorganization of the testis itself conditioned by the constant arrival of new germ-cells from some point outside the lobule. (4) The shortness of the time between

the close of spermatogonial multiplication and the first maturation division. Certainly if there is any increase in size in the primary spermatocyte in such forms as *Umbra* it is very slight as compared with the increase found in the primary spermatocytes of most animals. The spermatogonial growth period is accompanied by very little mitotic activity of the cell. It is initiated at the time the migrating cells assume a resting position in the lobule and persists up to the first week in August or thereabouts, although small clusters of isolated cells in a growing condition may, as already indicated, be found throughout the entire cycle of spermatogenesis. The large cells of the spermatogonial growth period and the cells resulting from their first division are the most favorable for chromosome study. Such cells (Fig. 2) in a resting condition may show one to three chromatin nucleoli. The chromatin granules are more centrally arranged than in the migrating cells (Fig. 1) and scattered along the linin network. The reticulum (Fig. 2) appears more compact than in the early migrating cells. These early growing or resting cells show (Fig. 2) from eighteen to twenty-one small compact bodies which react deeply to nuclear stains and are scattered throughout the reticulum. Their relative constancy in number and the fact that they correspond very closely to the number of chromosomes found in the spermatogonial cells, suggests the possibility of their being prochromosomes. They appear in the early resting or growing stages of the cell as deeply staining bodies scattered throughout the reticulum. In a very early prophase, as the chromatin granules begin to collect along the linin threads, these bodies seem to be the centers (Fig. 3) toward which the migrations of the chromatin granules progress. A somewhat later prophase shows the chromatin aggregated along the linin threads in a beaded condition. Although larger chromatin centers may still be seen, the beaded structure (Fig. 4) of the forming spireme in the middle prophase, makes further identification of these bodies as possible prochromosomes uncertain. The evidence goes to show, however, that these chromatin bodies are relatively constant as to number and are aggregation centers for the future chromosomes. No cells were observed in which such bodies could be seen in the

reconstruction period following the telophase in the dividing spermatogonia. All traces of the chromatin nucleoli, as such, have disappeared by the time of a mid-spireme formation. In the late spireme the beaded appearance of the thread disappears although its outlines remain rough up to and including the early prophase chromosomes. The spireme breaks up into definite chromosomes, twenty-two in number.

By the time the spermatogonial growth period has reached its height a great number of degenerating spermatogonia may be seen throughout the testis. These degenerating spermatogonia, and later, degenerating early spermatocytes, may be found in lobules in considerable numbers up until the time the lobule becomes filled with spermatozoa, but not later.

The prophase chromosomes of the spermatogonial divisions in *Umbra* are very long and thread like, and irregular in outline. No definite equatorial plate seems to be formed. Agar ('11) found a similar condition to exist in the spermatogonial cells of *Lepidosiren*. Subsequent metaphases or late prophases of these chromosomes show marked variation as to length, shape, thickness, and regularity of outline. Fig. 5 shows an early metaphase stage in which the chromosomes are all long and curved and mainly in the shapes of V's and L's; they clearly display differences in size, thickness, and shape. Still earlier, however, no such marked condition is to be seen, for the chromosomes are then all approximately of the same size, length, and thickness.

Figures 5 to 13 and 56 to 60 show progressive condensation changes in the chromosomes. These series of figures show that a gradual contraction takes place in all the chromosomes until a condition is reached like that shown in Figs. 12 and 13. Agar ('12), working on *Lepidosiren paradoxa*, found a similar condensation taking place in its chromosomes, the chromosomes contracting toward the apices of the V's. Figs. 12 and 13 show spermatogonial cells which exhibit this condition to a marked degree. Such contraction phases can be easily demonstrated in early spermatogonial cells because in this growth period such cells are very large, and the multiplications which take place are very slow. Later and smaller spermatogonial cells, in the

more active multiplication period, do not show such conspicuous gradations, although they do show the chromosomes to be in the shape of V's and L's. The writer believes that the condition as shown in Figs. 12 and 13 is unusual. Such figures are found in small numbers and usually occur at the periphery of a lobule or cyst and in small isolated clusters in the ends of the testis. The origin of the chromosomes in the form of V's and L's seems to be the more usual occurrence. The more unusual type of chromosomes have been of service in this study, however, since they furnish valuable material for checking chromosome counts made in other cells. Where the chromosomes appear as they do in Figs. 5 to 11, counting is difficult because they are not all in one plane. Some lie above the others, often in as many as three planes, and such an arrangement makes an accurate count often very difficult.

The chromosomes in the large spermatogonial cells are so clear that it is not difficult to pick out the homologous pairs. With this purpose in mind the chromosomes of Figs. 6 to 10, have been listed in Figs. 56 to 60 according to size. It will be noticed in all of these figures that although the chromosomes vary somewhat in shape at different times, two of them, labeled (*a*) in Figs. 6 to 10 and Figs. 56 to 60, although differing in size in the different cells have a fairly constant and characteristic shape. The members of this pair may be found side by side but more commonly one or two other chromosomes intervene. The fate of the chromatin nucleolus has not been fully determined in this study. Other investigators have shown that certain so-called nucleoli really constitute the sex chromosomes. Painter ('24), says: "It is concluded that the chromatin nucleolus is made up of the sex chromosome element in the opossum and presumably in all other mammals." The nuclei of the spermatogonial growth period and those at the end of the multiplication period (Figs. 15 to 17) in *Umbra* may show chromatin nucleoli varying from one to three. One is of more constant occurrence (Fig. 15), however, and it is in only a minor number of instances that two nucleoli are found in those spermatogonial cells which go into the maturation divisions. The relative constancy in size and shape of the two chromosomes just referred to, in all the spermato-

gonial cells studied, points to the possibility of their being sex-chromosomes, although no more positive evidence than this can be presented at this time. If they are sex-chromosomes the inference is that they would bring about the poultry type of sex-linked inheritance in *Umbra;* in other words the male would be of the two X, the female of the single X type. The condition here seems to be much the same as that depicted by Agar ('11), in *Lepidosiren;* according to his figures 18 to 31 two large chromosomes almost identical as to size and shape can be followed through the maturation division of the first spermatocyte, although he does not indicate that they are sex-chromosomes.

3. *Multiplication.*—Multiplication takes place to a small extent throughout the spermatogonial growth period. The new cells produced by these divisions grow somewhat and probably take part in the formation of some of the cysts within the lobule. If the first divisions of the early migrant cells are considered as the beginning of the multiplication period, then this period is a prolonged one. About the middle of July, however, active multiplication of the cells in some cysts is in progress. Such multiplication in *Umbra* is very rapid, once the reorganization of the lobules of the testis has taken place. Practically all of the lobules have completed their reorganization by the beginning of the first week of August. As before mentioned this reorganization of the lobules into cysts takes place not only through an enlargement of the migrating cells but also to some extent through their multiplication. Though isolated cysts in a lobule may still show cells in the spermatogonial growth-phase, the great majority of the cysts within any one lobule have practically or entirely completed their multiplication and are ready for the first maturation division by August. On the other hand a goodly number of cysts within the lobule have, by that time, completed the entire cycle of spermatogenesis. Turner ('19) says of the perch, "As each spermatogonium gives rise to a group of descendents they form a cyst and all pass through the same stages of division at the same time"; and this is in the main also true of *Umbra.* If one considers a cyst in *Umbra* as the product of a single spermatogonium, then an estimate of the number of spermatogonial divisions which have occurred in any one cyst

may be arrived at by counting the number of spermatogonia in a cyst which is ready for the first maturation division. On such a basis there are at least six generations.

The cells of the late multiplication period are usually so small and the chromosomes so massed that counts of the chromosomes are not satisfactory. In so far as counting can be done the numbers check with those obtained in the earlier cells where counts were possible. Although twenty-two chromosomes may be observed it is practically impossible to identify homologous chromosomes in these cells.

Turner ('19) describes in the perch a nuclear contraction of the transforming spermatogonia accompanied by the extrusion of liquids or particles into the cytoplasm. In *Umbra* no such phenomena occur. In all spermatogonial mitoses a definite centrosome appears at each pole of the spindle. The early spermatogonia of the spermatogonial growth period (Fig. 2) are from three to six times as large as the resultant spermatogonial cells of the last multiplication (Figs. 15 to 17) division. For example; the average early growth-cells range in diameter from 31.20 micra to 66.30 micra, while late spermatogonial cells which are ready for the first maturation division, have an average diameter of about 19.50 micra. While there are minor exceptions, the majority of the cells in the different cysts are approximately of the same size just before the first maturation division. This and the succeeding divisions are very rapid in *Umbra*. This is probably true in most of the Teleosts, and may be the reason why so many investigators have found difficulty in finding the various stages in fish spermatogenesis. It is necessary to cut and examine a very large number of sections in order to find the proper stages. In this study over five hundred slides were made and examined.

The First Maturation Division.

1. *Growth of the Primary Spermatocyte.*—At the time of the transformation of the spermatogonia of the last multiplication division (Figs. 15 to 17) into primary spermatocytes the spermatogonial cells may show one to three deeply staining chromatin nucleoli. The nucleoli apparently separate into chromatin granules.

During the transformation of such spermatogonia into spermatocytes they undergo an increase of 15 to 20 per cent. in size by the time they have attained the late pachytene stage.

2. *Leptonema, Pachynema and Zygonema.*—At the beginning of maturation, the chromatin nucleoli seen in the spermatogonia disappear. The leptotene threads are formed by a condensation of the chromatin granules on the linin network of the nucleus. The nucleus becomes filled with a mass of fine intertwining threads (Figs. 18 and 19) of which it is impossible to determine the exact number. From this step the succeeding stages of presynizesis follow each other in rapid succession. Fig. 20, an early zygonema, shows some irregular pairing of the threads. Figs. 21 and 22, judging from the increasing proportion of thick strands, show further advancement in the pairing of these threads. Fig. 23 represents a late zygonema or early pachynema in which the fusion has been completed in the mid regions of the threads, but in which the ends still remain single. Fig. 24 shows a stage, the pachynema, in which the fusion has been completed and a definite spireme is formed containing approximately one half the number of threads of chromatin found in preceeding stages. Although a definite count of these threads has not been attempted, by comparing this stage with the previous leptotene stage one can readily see it displays fewer threads.

Synizesis and Diakenesis.—Soon after the formation of the spireme of pachytene threads, the latter begin to concentrate toward the periphery of the nucleus (Fig. 25), leaving the center clear, with only a few chromatin strands connecting the peripheral masses. This massing of the pachytene loops around the nuclear membrane marks the beginning of synizesis (Fig. 25). There seems to be a definite tendency for the chromatin material to concentrate (Fig. 26) into one or the other of two areas at opposite sides of the nucleus, thus indicating a marked polarity. Fig. 27 shows a still more advanced stage of synizesis in which the chromatin is collected in the form of loops, principally toward one pole of the nucleus. The chromatin strands at the opposite pole are concentrated and do not show the looped condition. Two loops or rings (Fig. 27) can commonly be identified in this

stage at one side of the two main chromatin masses near the nuclear membrane. These two rings are connected by a thin strand of chromatin material. They apparently lose their identity in the following stage. Fig. 28 shows a further contraction phase. In this stage the chromatin masses at each pole draw away from the nuclear membrane and collect toward one side of the nucleus. Fig. 28 shows the remaining portion of the smaller mass being incorporated into the main mass. This marks the end of the period of synizesis.

Immediately following synizesis the nuclear membrane disappears. Fig. 29 shows the unravelling or breaking up of the chromatin knot into bars and smaller masses of chromatin. At this point eleven irregular chromatin bodies corresponding to the reduced number of chromosomes can be recognized. Figs. 30 and 31 show a further unravelling of these knotted masses or bars of chromatin. Rings or loops are to be seen appearing from the denser masses as shown in Fig. 29. These stages (Figs. 29 and 30) mark the final passing of the stage of synizesis.

The rings of chromatin which are to be seen in late diakenesis appear to be derived in two ways from the synizesic mass: (1) Long threads of chromatin segment after the unravelling of the previous stage and may be seen in twos (Fig. 61—*n* and *o*) one wrapped around the other, having the appearance of a definite strepsinema which might permit of a "double or triple cross over." Typically these threads apparently unwrap, however, and later condense into rings, each pair constituting such a ring. (2) Shorter segments may be seen in early stages following synizesis which arrive at the ring stage by a sort of unravelling or pulling apart as shown in Figs. 32 and 33, and (*a*) and (*b*) of Fig. 61. This pulling apart appears to take place at the ends first and later, a space appears between the two threads at the middle (Fig. 61—*c*, *d*, *e*, *f*); the ends having come into contact again.

Figure 61 (*a*) to (*p*) shows various diakenetic figures in the contraction and formation of the rings; these may have been derived from either condition previously described. Figs. 31 to 35 show various stages in the formation and further contraction of these rings. In Fig. 34 several chromatin figures in the form

of Y's are to be seen. These may have been produced either by the cutting across of some of the previously described loops, or they may be due to a further contraction of the loops in which the bivalents at one end of the Y have entirely united while the other end still shows the split which may be observed in the various diakenetic figures previously cited. The double split indicating the tetrad nature of these chromosomes, is not evident until they come upon the metaphase spindle of the first maturation division where end views can be secured (Fig. 36). At this stage, however, the tetravalent nature of the chromosomes is readily observable after proper staining and destaining methods. No irregular behavior, such as lagging or irregular divisions (Fig. 37) in early or late anaphase, was seen in these chromosomes. They pull apart forming dyads (Figs. 39 to 41), eleven in number in each daughter cell or secondary spermatocyte.

The Second Maturation Division.

The secondary spermatocytes (Fig. 38) are about one-third smaller than the spermatogonia which entered the first maturation division. Although resting nuclei (Fig. 38) are formed not infrequently, in secondary spermatocytes, the more common method of procedure is for the telophase of the primary spermatocyte to become rearranged immediately into the metaphase of the secondary spermatocyte. Both Geiser ('24) working on *Gambusia*, and Agar ('11) on *Lepidosiren* found likewise that no resting stage occurs between the telophase of the primary and the metaphase of the secondary spermatocyte. The writer believes that the condition found depends mainly upon the rapidity with which cell-division is going on. When the rate is slow a resting stage will be found but when rapid it is dispensed with. In *Umbra* the transition period between primary and secondary spermatocytes as well as the time of the secondary spermatocyte division itself is usually very brief. Figs. 39 to 41 show such a rearranged metaphase in which the chromosomes retain their definite bivalent condition. They still show their dyad nature plainly when at the equator (Figs. 39 to 41) of the spindle for the metaphase of this division. At a somewhat later time (Figs. 42 and 43) they tend to fuse or adhere to one another

so closely that they cannot be counted. The late metaphase or early anaphase spindles (Fig. 43) viewed from the side exhibit no unusual behavior of the chromosomes. No odd or lagging chromosomes are to be seen. The dyads are halved or pulled apart apparently along the longitudinal split indicated in the tetrads of the previous primary spermatocyte, and eleven small univalent chromosomes go to each daughter cell or spermatid. Fig. 44 shows a late anaphase in which chromosomes may be seen in a univalent condition.

Nuclear Changes Occurring During Transformation of the Spermatid.

The resting spermatids are approximately one half the size of the secondary spermatocytes. Only the nuclear behavior of the transforming spermatid will be considered. No attempt has been made to work out in detail the part taken by the cytoplasm or centrosomes in the process. A prolonged period ensues between the resting phase of the spermatid (Fig. 45) and the mature spermatozoan. At the beginning of spermiogenesis the chromatin collects along the linin of the nucleus in a rather scattered condition in which roughly seven to eleven chromatin masses (Fig. 46) may be distinguished. At the same time this initial condensation of the chromatin masses on the linin network of the cell is occurring, the axial filament begins to form. The tail, developed to about its full length, is present shortly thereafter. As the chromatin masses appear on the linin of the nucleus, their behavior is indicative of the formation of chromosomes for the metaphase of another division but before this process goes far the chromatin begins to migrate toward the periphery of the nucleus (Figs. 46 and 47). At this stage, scattered around the nuclear membrane, may be seen roughly five dense chromatin masses (Figs. 47 and 48), about twice as large as those which were visible in the more diffuse condition. These chromatin masses, up to and throughout this phase, remain connected by lighter staining strands with the linin of the nucleus. The appearance is that of a fusion of the smaller chromatin bodies of the previous stage in pairs. Following this condition there is a dense massing of the chromatin material

around the nuclear membrane (Figs. 49 and 50), leaving the center of the nucleus clear with only a few linin fibers connecting the masses at the periphery. Immediately following this peripheral arrangement of the chromatin in the form of a dense anastomosed ring (Figs. 50 and 51) beneath the nuclear membrane and around the clear center to the nucleus, a contraction of the nucleus takes place. Figs. 52 and 53 show final stages in the arrangement of the nuclear material to form the head of the spermatozoon. The heads of the spermatozoa formed by this contraction are somewhat smaller than the original spermatid nucleus. The heads of the mature spermatozoa are practically spherical (Fig. 55) although some may show such shapes as the one pictured in Fig. 54.

Discussion.

The literature on the spermatogenesis of the fishes or fishlike vertebrates is very limited. There is apparently no detailed piece of work relating to the Teleosts.

The prevalent opinion on the origin of the germ-cells in spermatogenesis is that they are formed either from cells which migrate into the testes, assume a fixed position, and produce the ultimate crop of spermatozoa, or that they are derived from reserve germ-cells which have existed as such since the formation of the first or primordial germ-cells in early embryogenesis. Moore ('95), working on the spermatogenesis of several genera and species of Elasmobranchs, Rawitz ('99), working on Scyllium canicula, Cunningham ('86), ('91), in two papers on the spermatogenesis of the Cyclostome (Myxine glutinosa), presuppose the origin of the primary spermatogonia from a germinal epithelium. Upon the spermatogenesis of the fishes proper, there are the papers of Agar ('11), ('12), on the spermatogenesis of *Lepidosiren paradoxa*; Turner ('19), on the seasonal cycle in the spermary of the perch; Geiser ('24), on the spermatogenesis of *Gambusia holbrooki*; and Winge ('22), on the cytology of Lebistes reticulatus. Winge does not discuss the spermatogonia, Agar assumes a development from a definite germinal epithelium, while Turner and Geiser assume an origin from migrating cells. Turner ('19) shows that in the perch the cysts are formed anew each season

and attributes the formation of the early spermatogonia to cells which migrate into the testes from a cord of germ cells outside. Geiser ('24) says: "The definite sex-cells by repeated divisions give rise to the final spermatogonia . . . . The cysts of spermatogonia are the result of the continued fission of the germ-cells which at the season of sexual activity migrate to the periphery of the gonad." A recent article on the origin of the germ-cells in the adult salamander is of special interest here. Hargitt ('24), working on the salamander (*Diemyctylus viridescens*) shows that the cysts do not retain residual spermatogonia, but the new crop of spermatogonia is formed each season from migrating cells of the peritoneal covering of the testis or from the epithelial lining of the collecting ducts. Hargitt found isolated nests of germ-cells in the mesenteries and peritoneum outside the testis but he does not attribute the origin of the new spermatogonia in old cysts to these cells. From his observations on the behavior of the germ-cells in the testis of *Umbra* the writer is led to believe that, as in the perch (Turner, '19), and in the salamander (Hargitt, '24), the primary spermatogonia are formed anew each season from cells which migrate from some point outside the lobules and possibly outside the testis. No cords of germ-cells were found outside the testis of *Umbra*, however, as described for the perch by Turner. The evidence points more to some such type of origin as that described by Hargitt for the salamander.

I find the seasonal cycle in *Umbra* to coincide in the main with that found in the perch by Turner ('19). Two distinct differences might be pointed out: (1) Beginning with the multiplication period the spermatogenetic cycle in *Umbra* is more rapid than that found in the perch. (2) The writer believes the migration of the new germ-cells to be a continuous process in *Umbra* though slower at some times than at others, instead of being restricted to any one particular period as was found in the perch.

The growth phase of the primary spermatogonia varies in different fishes. Turner ('19) working on the perch, and Geiser ('24), working on the top-minnow (*Gambusia*), found a definite growth period. In the perch the primary spermatogonia are

about three times the size of the final spermatogonia and in *Gambusia*, eight times the size of the final spermatogonia. It was found in *Umbra* that they are from three to six times the size of the final spermatogonia. Agar ('11) differentiates between primary and secondary spermatogonia in the Dipnoan, Lepidosiren. He finds that there is a decrease in nuclear size from thirty micra to seventeen micra during the transition from primary to secondary spermatogonia.

In the Dipnoi the final spermatogonia are only about one third the size of the primary spermatocytes. In this respect the Dipnoi appear to resemble the Amphibia more than they do the Teleostomi. It would be interesting to know what the condition is in the Elasmobranchs and Cyclostomes. The writer believes that this early growth phenomenon in the early spermatogonia in *Umbra* and other teleosts is bound up with the seasonal cycle in these forms; namely, the formation of spermatozoa from all the spermatogonia, the absence of reserve germ-cells, the destruction of the cysts of the lobule at the end of spermatogenesis together with their reformation the following season, and the formation of the early spermatogonia from migrating cells.

The number of generations these early spermatogonia go through before the final spermatogonia are produced varies in different fishes. Turner ('19) finds, in the perch, that there are five or six generations, Geiser ('24), in *Gambusia* ten to twelve. In *Umbra*, at least six generations are passed through before the final spermatogonia are formed.

Chromosome numbers have been given for the following species:

	Male.	Female.
Perca flavascens (Turner, '19)	27	
Gambusia holbrooki (Geiser, '24)	35 or 36	
Lebistes reticulatus (Winge, '22)	46	46
Lepidosiren paradoxa (Agar, '11)	38	
Scyllium canicula (Moore, '95)	24	
Scyllium canicula (Rawitz, '99)	20 to 24	
Umbra limi	22	

The behavior of the spermatogonial chromosomes in the fishes has been stressed little. Agar ('11) ('12) goes into the matter in Lepidosiren in some detail. He finds, as I do, that the chromosomes of the newly formed equatorial plates are long and

in the shape of L's and V's. Many of these chromosomes go through a contraction at the apices of the V's until small transversely constricted chromosomes are formed. Discussing the contraction phenomenon, Agar ('12), says: "It becomes operative especially, but not solely, whenever the chromosomes are short in comparison with their length, as happens normally in meiosis and exceptionally in somatic tissues." I find in *Umbra*, as has already been stated, numbers of spermatogonial cells showing varying degrees of contraction; this contraction is apparently toward the apices of the V's, as Agar has already pointed out. Early metaphase or late prophase (Fig. 5) in *Umbra* may occasionally show very long chromosomes with little or no evidences of contraction. Different cells show late metaphases and early anaphases in varying degrees of contraction. Anaphases may be found, although quite rarely, with chromosomes similar to those represented in Fig. 14. A more contracted state than that shown in Fig. 5, is probably the normal condition arrived at in metaphase. Extreme contraction, as shown in Figs. 12 to 14, are found in medium sized cells embedded between larger cells, in the regions of the testes where most connective tissue abounds, and at the periphery of the lobules. This extreme contraction is, I believe, the unusual rather than the usual occurrence. This condition as well as those that have been previously described, I interpret as being due to relative growth rates of the cells. Cells dividing rapidly do not show contraction to any appreciable extent while those dividing more slowly and remaining in each phase of mitosis longer, exhibit it more markedly. The slow growth in the extremely contracted phase may be due to tension exerted upon such cells by the connective tissue surrounding them, or by the larger and more actively growing neighbor cells. Contraction, however, is to a certain degree a regular procedure, and the shorter chromosomes, as was found by Agar in *Lepidosiren*, contract first and most. In *Umbra*, however, contrary to the view of Agar, I am inclined to believe that these transversely constricted chromosomes play no considerable part in meiosis.

Some investigators have reported a definite growth period, others no such period, in the primary spermatocyte divisions of

the fishes. In the Dipnoans and Amphibia the primary spermatocytes are three times the size of the spermatogonia of the last multiplication division. Turner ('19) finds that a growth period does not occur in the perch. Geiser ('24) shows (Figs. 11 to 21) a definite increase in size in the primary spermatocytes of *Gambusia*. In *Umbra* there is unquestionably a slight increase in nuclear size over that of the preceeding stage. It is probably due to the fact that the primary spermatocyte stage is one in which the cells remain longer than they do in the multiplication or secondary spermatocyte phases. All spermatocyte stages may be equally rapid in the perch, and this may perhaps be the reason Turner found no increase in nuclear size.

Maturation has been infrequently studied in the fishes or the Elasmobranchs. Moore ('95) has given a very good account of the essential points in the maturation divisions of Scyllium canicula, considering the technique employed by the workers of his time. The method of reduction described differs in no essential manner from that recognized in late years as the orderly procedure in most animals and plants. After the conjugation of the leptotene threads and formation of a synaptic knot (synizesis) the chromosomes segment into long thick rods of chromatin which pair and form a closed ring type of tetrad. The subsequent separation of the parts of the tetrad is accomplished by a transverse split through the center of the loop. The second division is similar to the first, with the exception that there is no reduction, namely, the separation of the dyads by a transverse split. The dyads separate and twelve univalent chromosomes are distributed to each daughter cell or spermatid. It is generally believed now that what Moore describes as a "split" in the ring is the pulling apart of homologous chromosomes at their previous line of fusion. Rawitz ('99) working on *Scyllium* states that at the time of reduction there is no reduction of the chromatin number, but that there is a reduction of the chromatin material by disintegration. He finds fourteen to sixteen chromosomes in the primary spermatocyte and fewer and larger chromosomes in the secondary spermatocyte. It may be that Rawitz has confused the spermatogonial and spermatocyte divisions. Geiser ('24) finds that in the early stages of diakenesis the

tetrads are of the open ring type, later becoming closed, at which time all evidence of their tetrad nature is lost. In *Umbra* during diakenesis, the chromatin segments into blocks of varying thickness which first pair up and form open rings, but later contract and form closed ones (Fig. 61). A further contraction at the time of the metaphase shows these tetrads condensed until all appearance of their tetrad nature has disappeared. In fact, unless carefully scrutinized, they may be mistaken for dyads. Upon destaining, however, it can be readily seen that they are arranged at the metaphase as typical tetrads (Figs. 35 and 36). Fig. 37 shows such tetrads arranged on the spindle for the first maturation division. The condition in *Umbra* is very similar to that shown in Figs. 45 to 48 by Moore ('95) in *Scyllium canicula*. In *Umbra*, as in *Scyllium*, the fiber attachment is median (Fig. 37), and the tetrads separate into dyads, the separation occurring along a longitudinal axis similar in manner to the opening of two V's which have been placed in apposition to each other. Separation begins at the apex of the V's and ends at the basal region. Thus dyads are formed which go to the secondary spermatocytes.

The behavior of the chromosomes in the secondary spermatocytes is not as easily followed as in the primary because of the greater irregularity of the chromosomes in lining up at the metaphase and of their subsequent merging together (Fig. 42) soon after they assume definite form. Furthermore, the chromosomes are much smaller than in the preceding division. In very early metaphase (Figs. 39 to 41), however, they can be readily made out as dyads and can be seen to pull apart (Fig. 43), eleven univalent chromosomes going to each spermatid. Another factor complicating the study of the chromosomes of the secondary spermatocytes is their proneness to rearrange immediately into the metaphase of the secondary spermatocyte from the telophase of the primary spermatocyte.

Winge's ('22) Figs. 8*a* to 8*c*, on diakenesis in the oöcyte of *Lebistes*, indicates a method of tetrad formation similar to that found in *Scyllium*, *Gambusia*, and *Umbra*. Agar ('11) found in *Lepidosiren* that at the conclusion of synizesis the chromosomes appear in the form of rings which later separate at their ends

forming univalent chromosomes. Each such univalent member shortens and becomes transversely constricted. After dissolution of the nuclear membrane, according to him, the chromosomes unite at one end to form a long bar shaped (quadripartite) bivalent which bends on itself and forms a ring. In the metaphase of the first meiotic division one member of each bivalent goes to one daughter cell, the other to the other. If his observation is correct, this is an unusual type of reduction and is the only case reported for the fishes. In *Umbra* the reduction phenomena follow more closely the account given for *Scyllium* by Moore ('95). Although transversely constricted chromosomes do appear in *Umbra*, as described by Agar for *Lepidosiren*, they unquestionably occur in spermatogonial divisions in *Umbra* and never during the time of meiosis.

Summary.

1. The spermatogonia of *Umbra limi* are formed anew each season from cells which migrate from some point outside the lobule, possibly in some cases even from outside the testis.

2. Evidence points to the presence of definite pro-chromosomes in the early spermatogonia.

3. The somatic and spermatogonial number of chromosomes is twenty-two. Chromosomes, at different times and in different cells, may exhibit contractions of varying degrees. Extreme contraction simulating a transverse fission is unusual.

4. Two large L-shaped chromosomes, relatively constant in size and shape, are found in all spermatogonial cells. They may be sex-chromosomes. If so, the male of *Umbra* is of the XX type.

5. The multiplication divisions of the spermatogonia are in general very rapid, with the result that the final spermatogonia are only of from one sixth to one third the volume of the original ones.

6. The primary spermatocytes of *Umbra* show roughly an increase in nuclear size of 15 to 20 per cent. over the spermatogonia of the preceding multiplication division.

7. The chromosomes emerge from synizesis in the form of rods or blocks of chromatin of varying length and thickness.

8. During diakenesis tetrads of the closed ring type appear. Final tetrad-formation is accomplished by the contraction of such rings. Carefully stained preparations show clearly the quadripartite nature of the tetrads in advanced metaphase when viewed from one pole.

9. Transversely constricted chromosomes are found in *Umbra*, but they are limited to certain spermatogonial divisions and play no part in the maturation divisions.

10. The tetrads have a median fiber attachment and separate into dyads at the metaphase of the first maturation division by a pulling apart at the apices of the loops or V's.

11. The secondary spermatocytes may go into a resting stage ibut usually the telophase of the primary spermatocyte passes mmediately into the metaphase of the second maturation division. The separation of the dyads at the second maturation division is very rapid.

12. No unusually large or lagging chromosomes were observed in the first or second maturation divisions.

13. The nuclear material of the transforming spermatid goes through an apparent fusion of chromatin masses, with a subsequent peripheral condensation of the chromatin, and a contraction of the entire nucleus as the head of the spermatozoön forms.

BIBLIOGRAPHY.

1. **Allen, E.**
'16 Studies on the Cell Division in the Albino Rat. II. Experiments on Technique, etc., Anat. Rec., Vol. 10, pp. 565–589.

2. **Agar, W. E.**
'11 The Spermatogenesis of *Lepidosiren paradoxa*. Qrt. Jour. of Micr. Sc., Vol. 57, No. 225, pp. 1–44.

3. **Agar, W. E.**
'12 Transverse Segmentation and Internal Differentiation of the Chromosomes. Qrt. Jour. of Micr. Sc., Vol. 58, No. 230, pp. 285–289.

4. **Aida, T.**
'21 On the Inheritance of Color in a Fresh Water Fish *Aplocheilus latipes* Temminck and Schlegel, with Special Reference to Sex-linked Inheritance. Genetics, Vol. 6, pp. 554–573.

5. **Cunningham, J. T.**
'86 On the Structure and Development of the Reproductive Elements in *Myxine glutinosa* L. Qrt. Jour. of Micr. Sc., Vol. 26, No. 105, pp. 49–76.

6. **Cunningham, J. T.**
'91 Spermatogenesis in *Myxine glutinosa*. Qrt. Jour. of Micr. Sc., Vol. 33, No. 129, pp. 169–186.

7. **Geiser, S. W.**
'24 Sex Ratios and Spermatogenesis in the Top-minnow, *Gambusia holbrooki* Grd., BIOL. BULL., Vol. 47, No. 3, pp. 175–208.

8. **Guyer, M. F.**
'16 Studies on the Chromosomes of the Common Fowl as Seen in Testes and in Embryos. BIOL. BULL., Vol. 31, No. 4, pp. 221–268.

9. **Hance, R. T.**
'17 The Fixation of Mammalian Chromosomes. Anat. Rec., Vol. 12, No. 3, pp. 371–386.

10. **Hargitt, G. T.**
'24 Germ Cell Origin in the Adult Salamander, *Diemyctylus viridescens*. Jour. of Morph. and Physiol., Vol. 39, No. 1, pp. 63–111.

11. **Moore, J. E. S.**
'95 On the Structural Changes in the Reproductive Cells during the Spermatogenesis of Elasmobranchs. Qrt. Jour. of Micr. Sc., Vol. 38, No. 150. pp. 275–313.

12. **Painter, T. S.**
'24 Studies in Mammalian Spermatogenesis. III. The Fate of the Chromatin Nucleolus in the Opposum. Jour. Exp. Zoöl., Vol. 39, No. 2, pp. 197–227.

13. **Rawitz, B.**
'99 Die Theilung der Hodenzellen und die Spermatogenese bei Scyllium canicula. L. Archiv. für Mikr. Anat., Vol. 53, pp. 19–62.

14. **Schmidt, J.**
'20 The Genetic Behavoir of a Secondary Sexual Character. Comptes-rendus des travaux du Lab. Carlsberg, Vol. 14, No. 8.

15. **Turner, C. E.**
'19 The Seasonal Cycle in the Spermary of the Perch. Jour. of Morph., Vol. 32, No. 3, pp. 681–711.

16. **Winge, Ö.**
'22 A Peculiar Mode of Inheritance and its Cytological Explanation. Comptes-rendus des travaux du Lab. Carlsberg, Vol. 14, No. 17, pp. 1–9.

17. **Winge, Ö.**
'22 One-sided Masculine and Sex-linked Inheritance in *Lebistes reticulatus*. Comptes-rendus des travaux du Lab. Carlsberg, Vol. 14, No. 18, pp. 1–20.

EXPLANATION OF PLATES.

Unless otherwise indicated the figures on plates 1 to 3 represent a magnification of about 2160 diameters as they are reproduced. Figs. 7, 14, and 35 represent a magnification of about 1650 diameters. A camera lucida was used for drawing together with a 2 mm. oil immersion (Zeiss) and a No. 18 ocular (Zeiss). Drawings 51, 54, and 55, are from smears; the remainder, from sections.

PLATE I.

FIG. 1. Ameboid cell of the migration period.

FIG. 2. Resting cell of the spermatogonial growth period showing a prominent chromatin nucleolus and twenty-one deeply staining chromatin bodies.

FIG. 3. Spermatogonium showing seventeen chromatin bodies which appear to be aggregation centers for the chromatin granules.

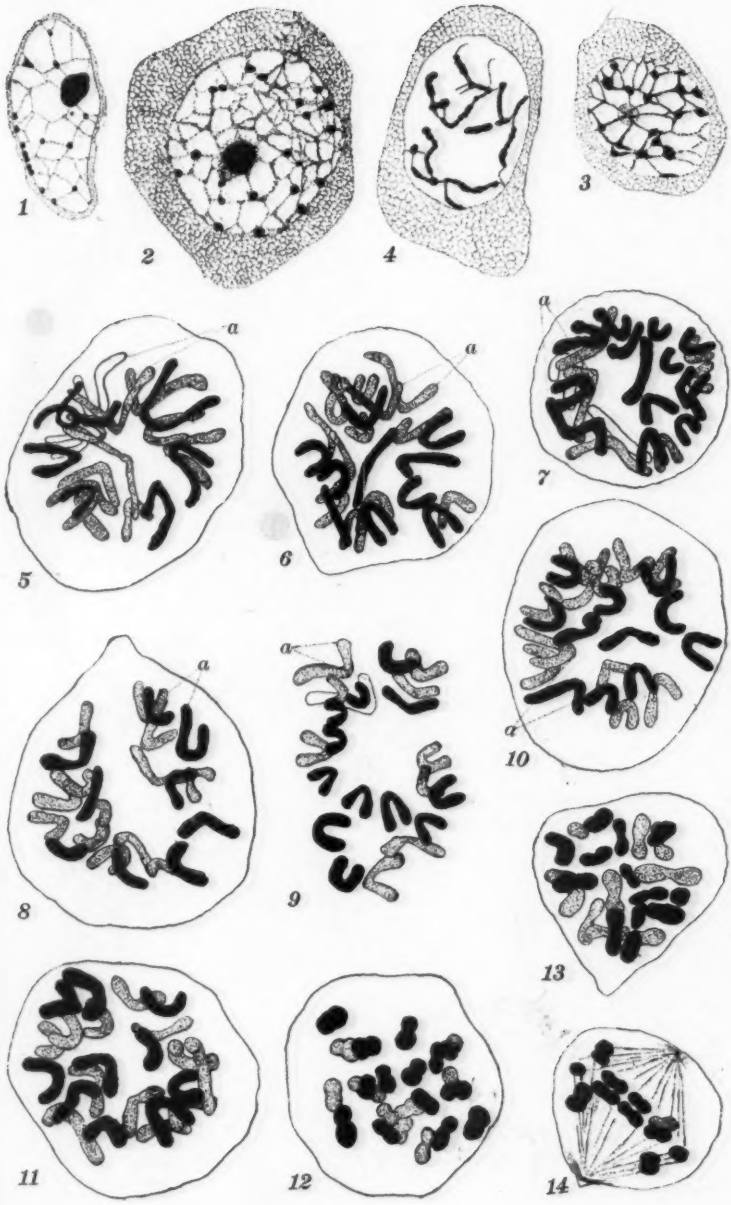
FIG. 4. Middle spireme of spermatogonial prophase showing the chromatin of the forming spireme in a beaded condition.

FIG. 5. Polar view of spermatogonial metaphase showing nineteen long and slightly contracted chromosomes.

FIGS. 6, 7, 8, 9, 10, AND 11. Polar views of spermatogonial metaphase showing twenty-two chromosomes in varying degrees of contraction. Probable sex chromosomes indicated at (*a*) in figures. Fig. 7 magnified 1650 diameters.

FIGS. 12 AND 13. Spermatogonial metaphase showing twenty-two transversely constricted chromosomes.

FIG. 14. Early anaphase of spermatogonium. Magnification 1650 diameters.



JAMES O. FOLEY.

PLATE II.

FIGS. 15, 16, AND 17. Resting spermatogonia of last multiplication division.

FIGS. 18 AND 19. Leptotene stages.

FIGS. 20, 21, AND 22. Zygotene stages.

FIG. 23. Late zygonema or early pachynema.

FIG. 24. Pachytene stage.

FIG. 25. Beginning of synizesis. The pachytene thread is massing around the nuclear membrane.

FIG. 26. Synizesis further advanced.

FIG. 27. Synizesis showing a looped condition of the chromatin.

FIG. 28. Synizesis completed. Complete contraction.

FIG. 29. Initial breaking up of synizesis. Only eight of the eleven chromatin masses are shown in figure.

FIG. 30. Final breaking up of synizesis.

FIG. 31. Continuation of unravelling process as shown in figure 30 and the beginning of diakenesis.

FIGS. 32, 33, AND 34. Various stages in diakenesis and contraction of the rings.

FIGS. 35 AND 36. Metaphases of primary spermatocytes showing tetrad nature of the chromosomes. Fig. 35 magnified 1650 diameters.

FIG. 37. Early anaphase of primary spermatocyte.

FIG. 38. Secondary spermatocyte in resting condition.

FIGS. 39, 40, AND 41. Early metaphase of secondary spermatocytes. Only six of the chromosomes are shown in figure 41.

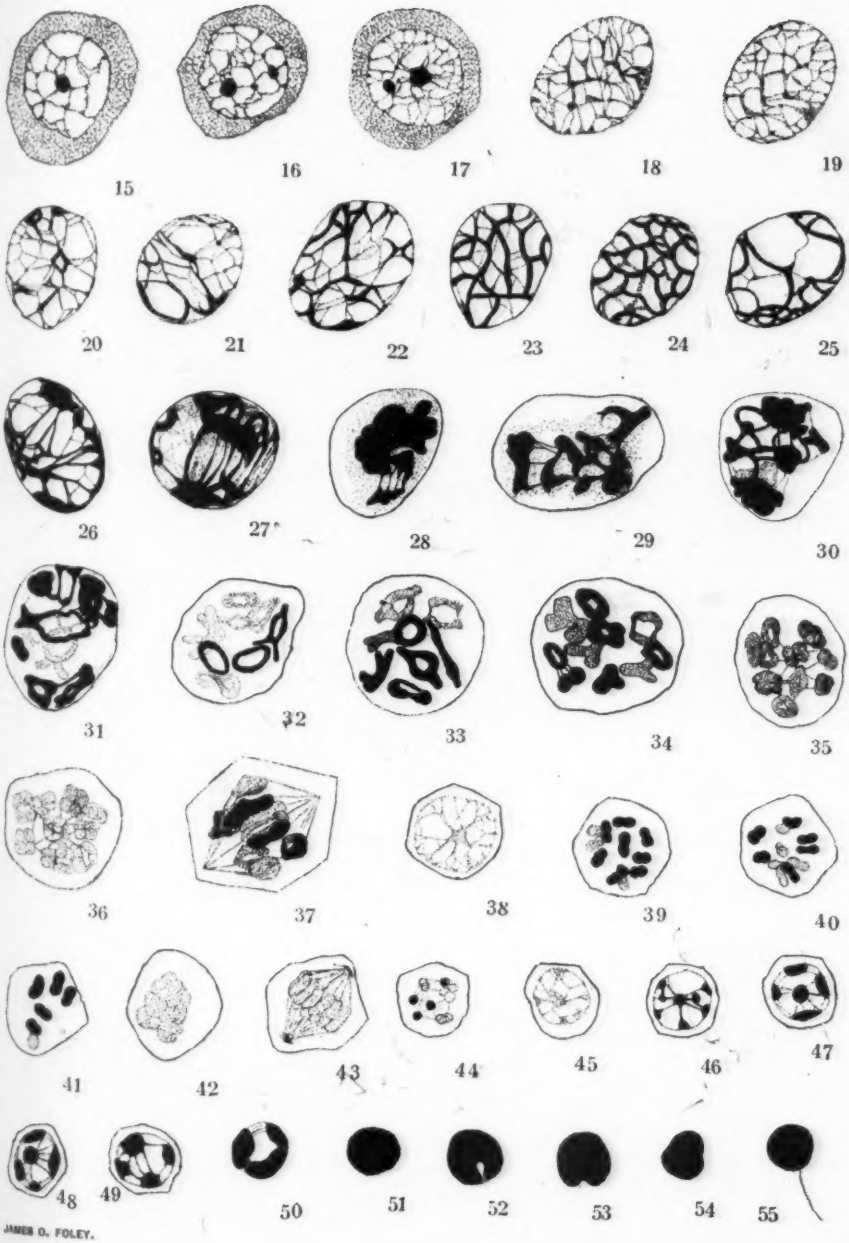
FIG. 42. Late metaphase of secondary spermatocyte.

FIG. 43. Late metaphase spindle of secondary spermatocyte.

FIG. 44. Anaphase of secondary spermatocyte showing eleven univalent chromosomes.

FIG. 45. Resting spermatid.

FIGS. 46 TO 55. Various stages in nuclear behavior during the transformation of the spermatid. Figs. 51, 54, and 55 are taken from smears. Condition shown in figure 51 is similar to that shown in figure 52 but viewed from a different angle.



JAMES O. FOLEY.

PLATE III.

FIG. 56. The individual chromosomes of Fig. 6.
FIG. 57. The individual chromosomes of Fig. 7.
FIG. 58. The individual chromosomes of Fig. 8.
FIG. 59. The individual chromosomes of Fig. 9.
FIG. 60. The individual chromosomes of Fig. 10.
FIG. 61. Various diakenetic figures selected at random from different primary spermatocytes.

JAMES O. FOLEY.

UNIV. OF MICH.

A STUDY OF THE GENETIC RELATIONSHIPS OF THE "AMEBOCYTES WITH SPHERULES" IN *ARBACIA*.

JAMES ERNEST KINDRED,

UNIVERSITY OF VIRGINIA MEDICAL SCHOOL.

The pioneer work of Geddes (1880) marks the beginning of the study of the cellular elements in the perivisceral fluid of echinoids by means of modern methods. He was the first to recognize the different types of cells, and his classification has been the basis of all subsequent studies. In recent papers I have reviewed the work of those who have studied the reactions of these cells in the living condition both in the Echinoidea and Echinoderms in general (Kindred, '21 and '24). In both of these papers my own investigations have been limited to the functional reactions of the leucocytes, with only incidental remarks as to the "amebocytes with spherules." From my observations I have concluded that the leucocytes are the most generalized cells of the perivisceral fluid and that they are highly phagocytic, thrombogenic and scleroblastic. The "amebocytes with spherules" apparently carry on none of these functions. To be sure, they cling to the clot when it is formed by the thrombogenic leucocytes, but their part is passive. Various investigators have sought to find out where the amebocytes with spherules arise and what they do. Thus far no unequivocal evidence has been presented regarding function, nor have specific places of origin been recorded for these cells.

Attempts to locate the source of origin of the amebocytes with spherules have led to the applications of blood stains to smears and sections, with a resulting classification of the cells by their tinctorial reactions. St. Hilaire (1897) was one of the first to use these stains successfully and he classifies the cellular elements of the perivisceral fluid of the echinoids by their respective tinctorial reactions as follows: *a*, small leucocytes with neutrophilic granules; *b*, larger leucocytes devoid of granules, but having one or more nuclei; *c*, amebocytes with basophilic

spherules; *d*, amebocytes with acidophilic spherules; and *e*, amebocytes with green spherules. Kollmann (1908), using the triacid stain of Ehrlich, following fixation with Zenker's fluid, does not find leucocytes with neutrophilic granules. Of the amebocytes with spherules, he studied only those with basophilic spherules. He states that these spherules are amphophilic. On comparing the tinctorial reactions of the several types of cells with their characters in the living condition, St. Hilaire interprets the basophilic spherules as identical with the colorless spherules of the living cell, the acidophilic as identical with the reds, and the green spherules as degenerate reds.

According to Kollmann the chemical composition of the colorless spherules is complex. They contain an albuminoid substance insoluble in alcohol and an alcohol-soluble lecithin. The red spherules seem to be of a more complex albuminoid nature and also contain a lecithin. The albuminoid nature of the spherules of those cells with colorless spherules accounts for their relatively slow rate of movement and also for their greater number in the connective tissue as contrasted with their number in the perivisceral fluid. Since the mast cells of vertebrates exhibit comparable tinctorial reactions and connective tissue affinities, Kollmann infers that the amebocytes with colorless spherules are comparable with them.

Kollmann also states that although he can find no relation between leucocytes and amebocytes with spherules, it is possible that the latter arise from the former by the development of spherules within the hyaline cytoplasm.

The following investigation has been undertaken to determine a possible source of origin for the amebocytes with spherules from leucocytes. The inspiration for this investigation came from the suggestion by Cuenot ('91) that the spherules are extrinsic in origin, nutritive in nature, and that the amebocytes with spherules may arise from leucocytes which have ingested food droplets.

Material and Methods.

Examinations were made of fresh drops and fixed smears of perivisceral fluid of Arbacia punctatum. Some of the smears were fixed in Helly's fluid and stained either with the Giemsa

stain or with Ehrlich's triacid blood stain; others were fixed and stained by Wright's method; and those of a third group were fixed in the fumes of 2 per cent. osmic acid. By far the best and most uniform results were obtained with the Helly-triacid preparations. In addition to the smears, parts of the intestine were fixed in Helly's fluid, embedded in paraffin, sectioned at 7 microns, and stained with Ehrlich's triacid stain or with eosin-azure II. The eosin azure gave the most satisfactory results.

Observations.

Fresh Perivisceral Fluid.—The cellular elements present in the fresh perivisceral fluid of Arbacia have already been described in detail (Kindred, '21), hence a brief description of these cells will suffice here. The leucocytes are small, have a granular endoplasm containing clear vacuoles (acid to neutral red), and bear varying numbers of flap-like pseudopodia (Fig. 1). These cells have been observed to be phagocytic, thrombogenic and scleroblastic.

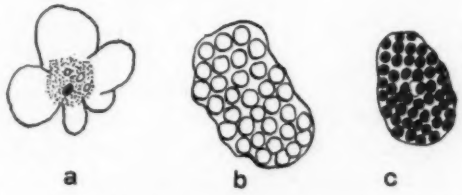


Fig. 1. Cells from fresh perivisceral fluid. *a*, leucocyte; *b*, amebocyte with colorless spherules; *c*, amebocyte with red spherules. × 1200.

Amebocytes containing either colorless, red, or yellow spherules are present in all drops of perivisceral fluid (Fig. 1). The amebocytes with red spherules are the most numerous, those with colorless spherules next, and those with yellow spherules least numerous. None of these cells have been observed to be phagocytic, thrombogenic, or scleroblastic. They move by means of blunt pseudopodia. These cells possess distinct cell membranes. The colorless spherules are larger than either the red or yellow spherules which are approximately of the same size. The nucleus of an amebocyte appears as a light area near the center of the cell.

Fixed and Stained Smears of Perivisceral Fluid.—In smears fixed with Helly's fluid and stained with Ehrlich's triacid stain, a very clear differential staining of certain cells occurs. The leucocytes are only slightly affected by the stain. No distinct granulations are present in the cytoplasm. The nucleus is, relatively large as compared with the cell body (Fig. 2).

The spherules of the amebocytes with colorless spherules are amphophilic to basophilic and stain violet to blue. Furthermore, these spherules do not retain their spherical shape (Fig. 2). The nucleus of this type of cell is small and compact as compared with the size of the cell, but it is actually as large as the nucleus of the leucocyte.

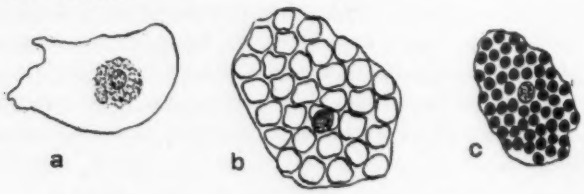


FIG. 2. Cells from smear of perivisceral fluid stained with Ehrlich's triacid stain. *a*, leucocyte; *b*, amebocyte with basophilic spherules; *c*, amebocyte with acidophilic spherules. × 1200.

The red spherules are acidophilic in reaction and stain a brilliant red (Fig. 2). These spherules retain their spherical shape, and are as sharply distinct in the fixed, as in the living material. Some of these spherules exhibit stages of degeneration and stain less strongly than others, a fact which leads to the inference that they may possibly be the spherules of amebocytes with yellow spherules, since the latter present no other definite tinctorial reaction. The relative number of cells with faintly acidophilic spherules corresponds in general with the number of amebocytes with yellow spherules in the living condition.

In smears fixed in the fumes of 2 per cent. osmic acid the spherules of the amebocytes are preserved and are sharply outlined in black. The color differences between the spherules are lost. The positive reaction of the spherule membrane to the osmic acid as contrasted to the solubility of spherule content is interpreted to mean that the albuminous part of the spherule is its content, and that the lecithin part forms the membrane.

Sections of the Alimentary Canal.—Transverse sections of the wall of the esophagus show a lining epithelium of tall and extremely narrow cells. This epithelium extends nearly across the whole thickness of the wall. Below the epithelium is a thin layer of connective tissue, reticular in character. This layer is invested by a thin tunica muscularis, superjacent to which is a delicate peritoneal epithelium. A similar relationship of layers exists in the wall of the intestine proper (Fig. 3), but the con-

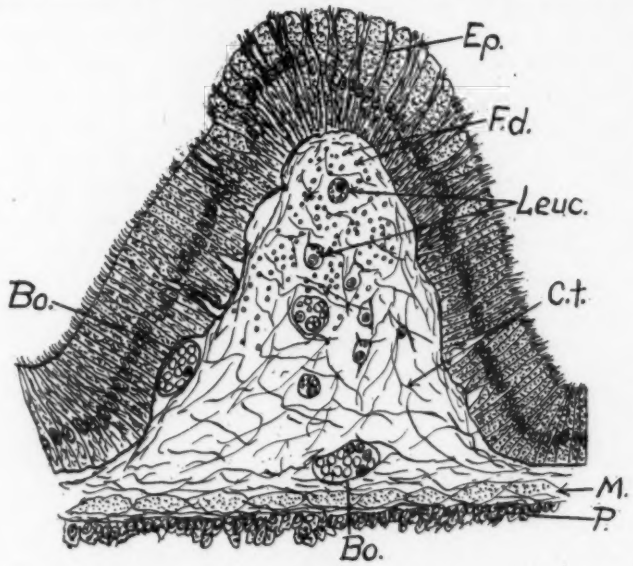


FIG. 3. Transverse section through part of intestinal wall. *Bo.*, amebocyte with basophilic spherules; *C.t.*, connective tissue layer; *E.p.*, epithelium; *F.d.*, food droplets; *Leuc.*, leucocytes; *M.*, muscular layer; *P.*, peritoneum. × 750.

nective tissue layer is much thicker and thrown into folds which are followed by the epithelium. At the base of each connective tissue fold there are several lacunæ. In the meshes of the connective tissue strands there are numerous minute greenish droplets. From the positional relationship of these droplets to the intestinal epithelium, I infer that they are food droplets. From the extreme minuteness of the droplets nearest the basement membrane it seems that they have passed through the

cell membrane and basement membrane in fluid form not as droplets. Having reached the connective tissue the fluid is dispersed in the form of droplets.

Among the food droplets large numbers of leucocytes can be seen. Most of these leucocytes are in the act of ingesting droplets. The ingested droplets vary in number and in size (Fig. 3). In those cells which have ingested large numbers of droplets the cell body is distended far beyond the normal size of a leucocyte. The cells containing the most droplets are, as a rule, nearer the peritoneal surface of the intestinal wall. During the first stages of ingestion the droplets are smaller than the spherules of the amebocytes, but during the later stages of ingestion, the small droplets coalesce to form larger ones. The color of the droplets all through the first stages of ingestion is greenish and the droplets are apparently unaffected by the stain. As the droplets enlarge they acquire a slight basophilic reaction.

Cells with typical basophilic spherules are very numerous in the lacunæ at the bases of the regions of food ingestion and sometimes are found in the connective tissue meshes among the ingesting leucocytes. Amebocytes with acidophilic spherules, on the other hand, are seldom present in the intestinal wall.

Conclusions.

One phase of this investigation has demonstrated that the tinctorial reactions of the cells of the perivisceral fluid of *Arbacia punctatum* are identical with those of other echinoids. That is to say, the cytoplasm of the leucocytes is not tinctorially active, the spherules of the amebocytes with colorless spherules are basophilic in reaction, and the spherules of those with red or yellow spherules are acidophilic in reaction.

The second phase of this investigation reveals what I infer to be a genetic relationship between the leucocytes and the amebocytes with spherules. This inference depends primarily upon the interpretation placed upon the tinctorial reactions of the food droplets ingested by leucocytes in the intestinal wall. My preparations show leucocytes ingesting food droplets. It is also known that the leucocytes contain vacuoles giving an acid reaction to neutral red (Kindred, '21). Hence those cells in

which the droplets begin to exhibit a basophilic reaction are probably those in which the acid content of the vacuoles has begun to act upon the droplets. Further action of this acid or digestive enzyme renders the cell one in which the droplets, now in the form of larger spherules, are distinctly basophilic in reaction. Thus amebocytes with colorless spherules are inferred to arise from leucocytes which have ingested food. Further evidence for this inference is given by the relatively great numbers of amebocytes with basophilic spherules in the intestinal wall in the region of food ingestion.

It is further possible to conceive that the colorless spherules undergo a gradual digestive change as the amebocyte containing them passes through the tissues of the body. Judging from the sequence of events in intracellular digestion in the Protozoa this change would be in the direction of acid to alkaline. Hence it is inferred that these colorless spherules, during the process of intracellular digestion, gradually give off part of their substance to the tissues through which the amebocyte containing them passes. Subsequently the spherules shrink in size and become alkaline in nature. This change in size and chemical nature could possibly be interpreted as the manner in which red spherules arise. The end product of the red spherules would be the yellows.

If my inference as to the genetic relationship between the amebocytes with colorless and red spherules is correct then the greater number of amebocytes with red spherules in the perivisceral fluid is caused by the accumulation here, near several avenues of escape, of degenerating cells. The idea that that the amebocytes with red spherules are degenerating cells is further borne out by observations which have shown that they pass out through the gills, or else they undergo crystalline degeneration in the test (List, '97).

BIBLIOGRAPHY.

Cuénot, L.

'91 Études sur le sang et les glands lymphatiques dans la série animale. Parti. 2, Invertébrés. Arch. Zoöl., sér. 2, 9, 613–641.

Geddes, P.

'80 Observations sur le fluide périviscéral des oursins. Arch. de Zoöl. exp. et gener., 8, 483–496.

Kindred, J. E.

'21 Phagocytosis and Clotting in the Perivisceral Fluid of *Arbacia*. BIOL. BULL., Vol. 41, 144–152.

'24 The Cellular Elements in the Perivisceral Fluid of Echinoderms. BIOL. BULL., Vol. 46, 228–251.

Kollmann, M.

'08 Recherches sur les Leucocytes et le Tissu Lymphoide des Invertébrése Université de Paris, pp. 240.

List, T.

'97 Über die Entwickelung von Proteinkrystalloiden in der Kernen der Wanderzellen bei Echinoiden. Anat. Anz., 14, 185–191.

St. Hilaire, C.

'97 Ueber die Wanderzellen in der Darmwande der Seeigel. Trav. Soc. Imp. Natur. Petersbourg, 27, 221–248.

A COMPARISON OF MITOSIS IN CHICK TISSUE CULTURES AND IN SECTIONED EMBRYOS.

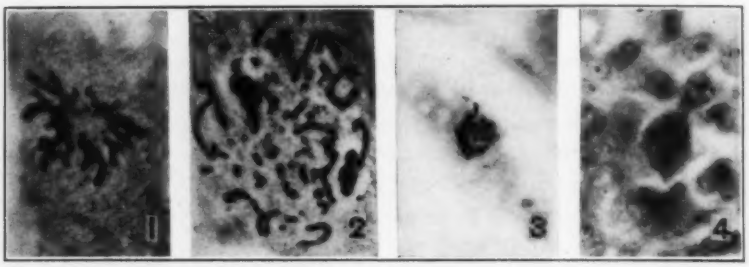
ROBERT T. HANCE,

National Research Fellow, University of Pennsylvania.

Those accustomed to working with tissue cultures are, I am sure, seldom troubled with doubts as to the essential normality of the processes going on in these isolated bits of tissue. Others, less familiar with the results and conditions of this type of work, usually make cautious reservations and distinctions in discussing phenomena occurring in living substance under its usual associations and when subjected to the technique of the tissue culture. Through the interest and courtesy of Mrs. M. R. Lewis of the Dept. of Embryology, Carnegie Inst. of Washington it has been possible for me to study mitosis in a number of beautifully fixed preparations of cultures of chick tissues. As this led later to an examination of this process in chick embryos a comparison was inevitable and a note concerning the characteristics of dividing cells as found in tissues existing under the two environments seemed desirable.

As regards fixation the tissue culture is infinitely easier to kill satisfactorily than a larger mass of tissue. The cells in the culture grow out over the cover glass usually in a single layer and the fixing reagent can come in contact with each cell individually and at once. This is, of course, an ideal which can never be approximated in material that must be sectioned. Consequently many fixatives which give excellent results with cultures are worthless when used on bulkier masses of cells. The size of the cells in the culture is another item in their favor for with the release from the crowded quarters of their usual surroundings they spread over the cover glass and press very closely to it, causing the diameter of the cell to increase greatly, associated with a marked thinning in the plane perpendicular to the cover glass. The cell becomes consequently much easier to see through and its various parts appears spread out in diagrammatic fashion.

There is also an increase in the size of the chromosomes as can be seen by comparing Fig. 1 with Figs. 3 and 4. This is an actual increase in size and may be caused by the increased or richer nourishment that it is possible to offer cells reared in cultures rather than by the mere flattening of the cell and its contents. It seems apparent, although but few accurate measurements have



Photographs of dividing chick cells. × 1000.

FIG. 1. Late prophase of mitosis of cell growing in tissue culture.

FIG. 2. Prophase of mitosis of cell in tissue culture. Note the extreme flatness of the arrangement of the chromatic particles.

FIGS. 3 AND 4. Polar views of metaphase plates found in sectioned chick embryos. Compare their dimensions with Fig. 1.

been made, that whatever has caused the chromosomes to increase in size has affected all equally. This observation is in harmony with the results reported elsewhere (Hance, 17 and 18) where it was demonstrated that an increase in the total amount of chromatin in a given cell was the result of a proportional increase in the size of each individual chromosome.

The prophase stages of division which under ordinary conditions are like a ball of threads are in the cultures spread out so flatly that every granule of chromatin in the nucleus can frequently be recorded by photomicrograph as readily as can be the chromosomes of a very flat metaphase plate (Fig. 2). As the cells in the cultures are so exceedingly thin division never takes place perpendicular to the cover-glass but by the time mitosis has reached metaphase the chromosomes rearrange themselves so that division occurs in the plane of the cover. Consequently polar views of the metaphase cannot be found in cells in cultures although the very late prophase is so similar that for purposes

of enumeration of the chromosomes it is quite satisfactory. Centrosomes and spindles are quite similar in cells dividing under the two conditions although they are clearer and possibly larger in the cultures.

An examination of the chromosome number has developed no distinctions between the two types of material. In the prophase of the culture cells the number of chromatic individuals is very large (from 60 to 70 in some cases). Prophases in which all chromatin rods or bodies can be counted are somewhat rare in sectioned embryonic cells but enough have been observed to indicate their entire similarity to the former. As the metaphase stage is approached the number of distinct chromosomes becomes less, presumably through the coalescence of certain chromatin granules or rods which previously had seemed to be separate. (See Fig. 2 for various series of chromating granules lying in straight lines which probably coalesce later in the mitotic process to form single chromosomes.) This results in a metaphase chromosome number that probably is between 35 and 40, it being difficult to determine the exact number owing to the minuteness of the smaller chromosomes. Nothing unusual develops in the culture anaphases and division is completed as normally there as in the embryo.

The tissue culture divisions were originally called to my attention by Mrs. Lewis because the chromosomes seemed to show some evidence of fragmentation such as I had reported finding in other forms (Hance, '17 and '18). Certain cells did possess a larger number of chromosomes than others. As already stated above the larger number is apparently an early prophase condition and the number decreases as the cell approaches metaphase through the union of what, in the prophase, have seemed to be discrete bodies. This condition occurs in embryos as well as in cultures. There is a little evidence drawn from instances where occasional somatic chromosomes are apparently without mates identical in form and size that suggests the possibility of fragmentation. However, considering the quite definitely determined condition discussed above of the running together of prophase granules to form chromosomes the asymmetry of the mates may as well, or perhaps even more likely, be due to the non-completion or total failure of the coalescing process as to fragmentation.

The retention of chromosome identity or individuality in the culture raised cells is also obvious. Fig. 5 shows a series arranged

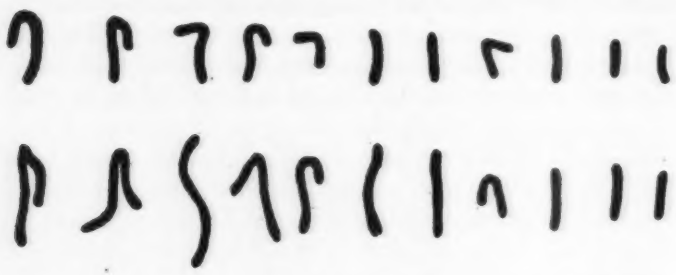


FIG. 5. Camera lucida drawings of the longer chromosomes of the chick complex arranged according to length.

Top row—from sectioned embryo.

Lower row—from tissue culture cell.

according to length, of the longer chromosomes of the mitotic complexes from cells found in a sectioned embryo and in a tissue culture. The smaller chromosomes of each cell have been omitted for convenience in printing and because, being short rods, they show no easily distinguishable features. A slight variation between the two sets of chromosomes illustrated enters through it being impossible, as pointed out above, to find a polar view of a metaphase plate in tissue culture cells. The lower series of chromosomes is taken from a very late prophase (tissue culture), so late that many of the chromosomes have assumed characteristic form. Number (from left to right) 3 has not yet taken on its final form. With this exception in mind it can be seen that the form of corresponding chromosomes in the two series and that the comparative size relations of a given chromosome to others in the same series or in the other series are identical. The possible causes for the chromosomes of the culture cells being larger have been discussed above.

It is possible then to conclude that tissue cultures, as far as the mitotic phenomena are concerned, are quite normal and differ from these processes in the body only in minor reactions to the physical limitations of their surroundings.

BIBLIOGRAPHY.

1. **Hance, Robert T.**
'17 The Diploid Chromosome Complexes of the Pig (*Sus scrofa*) and their Variations. J. of Morph., Vol. 30, 155–222.

2. **Hance, Robert T.**
'18 Variations in the Number of Somatic Chromosomes in *Oenothera scintillans* DeVries. Genetics, Vol. 3, 225–276.

THE IRON–ACETOCARMINE METHOD OF FIXING AND STAINING CHROMOSOMES.

JOHN BELLING,

CARNEGIE INSTITUTION OF WASHINGTON.

A short description of this procedure was given in Vol. 55 of the *American Naturalist*, pp. 573–574. The method has now been used almost daily for five years, and certain improvements have suggested themselves.

The solution can be prepared by mixing about equal volumes of glacial acetic acid and water. To this is added powdered carmine in excess. The liquid is then just brought to boil, cooled, and decanted. To the red solution there are then added a few drops of a solution of ferric hydrate in 50 per cent. acetic acid, to act as a mordant. The amount of iron varies with different objects. Too much produces a precipitate in a short time. The more iron, the darker and bluer is the stain. A bluish red is usually the best. Large cover-glasses (20 by 50 mm.) should be used, and they should not be thicker than 0.17 mm. (Small or thick covers prevent pressure being properly applied to individual cells.) There should be a minimum thickness of liquid under the cover-glass. The edges can be sealed with a honey-thick solution of dammar in xylol applied with a brush; or better perhaps, with melted soft paraffin.

The following varied modes of preparation are useful in different cases.

1. Pollen-mother-cells, etc., are obtained free by pressing or tapping cut segments of anthers in a drop of iron-acetocarmine.

2. The cut-up anthers, or minute fragments of animal ovaries or testes, are put in a tube with a *large* excess of iron-acetocarmine. After 2 to 7 days, preparations are made in the usual way. This gives good results with cancer tumors.

3. Smears of large anthers or testes are made on a cover-glass, which is then laid on a drop of iron-acetocarmine on a slide.

4. For staining the vegetative and generative nuclei in pollen-grains or pollen-tubes, etc., crystals of chloral hydrate are dissolved in a few drops of iron-acetocarmine until the liquid just

clears the specimens sufficiently. (Too much chloral hydrate will cause shrinkage.) The pollen-grains are squeezed or partly crushed in this liquid, and may be left to stain for a day or two.

FIG. 1. Hyacinth—$2n$.

In the case of pollen-mother-cells, local pressure, with a small roll of paper, on the cover-glass, often spreads out the cytoplasm on the cover-glass or slide, with the chromosomes uninjured. If required, these can be preserved in balsam, by replacing the liquid by graduated mixtures of 45 per cent. acetic and alcohol. Fig. 1 is a balsam preparation of the reduction metaphase in a hyacinth, prepared in this way.

FIG. 2. *Datura*—$2n$—1.

The cytoplasm becomes quite clear in the acetic acid, and with an appropriate green screen on the microscope, the bluish red chromosomes stand out jet black. (Wratten green films Nos. 66 and 56, are most useful on a binocular, and No. 58 on a monocular, with a good tungsten electric light.) Fig. 2, a pollen-mother-cell of *Datura* in the second metaphase, with 23 chromosomes, in iron-acetocarmine, shows the sharpness of the images.

Pressure is usually best applied from 2 to 7 days after mounting, but the time varies for different pollen-mother-cells. Well prepared slides will often keep for some months.

The following notes may be of use.

Canna.—The pollen-mother cells are large and tender. The anthers should be cut into segments about 1 mm. long, and gently pressed out in a large drop of iron acetocarmine, being left ten minutes or so to toughen before putting on the cover-glass. (Here, of course, as usual, the anther remains are previously removed.)

Zea.—In maize the pollen-mother-cells are delicate, and the same process is required as for *Canna*.

Nicotiana.—In tobacco the pollen-mother-cells are easily pressed out and stained.

Datura.—Resembles a small tobacco. Pollen hard to stain.

Hyacinthus.—Yields excellently stained pollen-mother-cells, and also pollen-grains. The latter require several days to stain well. Pressing out is difficult.

Triticum and Secale.—In wheat and rye all the pollen-mother-cells are connected and come out of the anther loculus in a string. The young anthers are cut across once, and pressed out under the cover-glass. The pollen-grains stain well.

Tradescantia.—Stains deeply and shows chromomeres well in pollen-mother-cells, and in pollen-grains.

Uvularia.—Shows chromosomes like those of the Orthoptera in the pollen-mother-cells. Pollen stains well. Some metaphase chromosomes of the reduction division show compound rings.

Hosta.—Shows large and small chromosomes, the former with marked chromomeres. Stains well.

Cypripedium acaule, and C. pubescens.—Both pollen-mother-cells and pollen-grains stain well. Ten pairs of chromosomes.

Scilla sibirica.—Pollen-mother-cells stain well. Shows six pairs of chromosomes.

Narcissus.—Pollen-mother-cells and pollen-grains stain well.

Galanthus.—Pollen-mother-cells stain remarkably easily and well. Several different sizes of chromosomes.

The above plants have chromosomes which are especially worth studying, because of their size and for other reasons. In *Brassica*, *Gladiolus*, *Iris versicolor*, *Asparagus*, *Dahlia*, *Capsella*, *Hemerocallis*, *Antirrhinum*, *Stizolobium*, *Phaseolus* and *Crocus*, the chromosomes are either small, or clumped and not easily unravelled.

STUDY ON THE HABITS OF THE CRAB *DROMIA VULGARIS* M.E.

W. S. DEMBOWSKA,

M. Nencki Institute for Experimental Biology, Warsaw.

The crab *Dromia vulgaris* usually carries on its back a sponge which it holds with the especially modified 4th and 5th pairs of ambulatory legs. The sponges, found on crabs at Villefranche, belonged to very different species. Only in the minority of cases did the sponge belong to the species *Suberites domuncula*, although the symbiosis of *Dromia* and *Suberites* has become nearly a classical instance. The sponges may belong to both the Calcarea and Incalcarea groups and very often *Dromia* covers its back with colonies of Ascidians, Algae, and even with pieces of paper or rags of any possible color. The fact of the non-specificity of this symbiosis was known to Polimanti and I believe that in reality there is no true *symbiosis* at all, as the sponge seems to be just as accidental a material as paper or rags. There is no doubt that all the activity is on the side of the crab.

The sponge ordinarily is astonishingly adapted to the size of the crab and to the shape of its back, especially in young individuals. It seems to be an exact copy of the back's surface. The cause of such a conformity is not exactly known. Renier uttered the hypothesis that the larva of *Suberites* becomes fixed on the back of a young *Dromia* and then both organisms grow together which accounts for the conformity of shape. However, the thing seems to be utterly improbable as there is no specificity in the "symbiosis." The pieces of paper may be just as well adapted to the size and to the shape of the crab as the sponge and thus even if the theory of Renier proved to be true, it cannot account for all the facts. Moreover the sponge is never fastened to the back, but it is simply held by the crab.

According to Vosmaer *Dromia* may tear off *Suberites* which are fastened to the convex shells of molluscs, obtaining in this way a concave surface that it puts then on its back. According to

my own observations, this statement is fairly correct. I might add that *Dromia* can do the same with any kind of sponge, fastened to any immovable object. But in this case the hypothesis of Vosmaer becomes not quite adequate—as the sponge, torn off from a large stone, may be perfectly flat and there will be no conformity in shape. As far as the writer is aware the process of cutting and tearing off of the sponge by *Dromia* has not yet been described. The following observations and experiments pour some light on the whole question and they demonstrate at the same time the complication and the variability of the behavior of the crab.

The Process of Manufacturing a Case.

The process of manufacturing a case by *Dromia* may be easily observed if we put in the aquarium, which contains a crab, a comparatively large piece of ordinary writing paper. Before putting in the paper we remove the sponge from the crab's back. The paper must be boiled for a few minutes in water, otherwise it would float.

As a rule very soon *Dromia* grasps the edge of the paper with its claws and begins the work. The crab enters under the paper, holds the edge tightly and turns with its back to the ground. Now by means of rhythmical movements of both chelæ the crab cuts out of the paper a piece which is fairly similar in size and shape to the removed sponge. Nearly always *Dromia* starts its work from the edge. To state it more exactly, the crab does not cut the paper, but it tears it, and therefore the edges of the new case are always very uneven. Both chelæ grasp the paper until their points nearly touch one another, at the same time their planes are perpendicular to the edge of the future case. At the next moment both points are directed forward (upward if the crab lies on the back) and the paper between them tears. At once *Dromia* grasps the paper a little farther and tears it again in the same way. The animal pursues its work systematically and it keeps working always in a definite direction. As the border of the resulting case is approximatively elliptic, the mutual position of the chelæ cannot be always the same, but it varies according to a definite rule. It often happens that the crab

pulls the paper too strongly and the edge becomes torn farther than necessary. But very rarely is the future case damaged in this way, nearly always such tears are directed outwardly. It is rather important to note that the animal does not pursue its work along every cleft but it chooses the right direction, correcting it every time. The clockwise direction of the work was just as frequent as the opposite one.

The legs of 2d–5th pairs participate indirectly in the work. They hold the paper and present it to the chelæ. If the crab does not stop in its business, which occurs from time to time, the process of cutting a case lasts about 15–30 minutes on the average.

The mode of working described is very frequently to be observed; however, it is not the only one possible. Sometimes before the cutting of the case is finished the crab grasps it with the hooks of the 4th–5th legs and puts it on the back. The case remains bound to the main mass of the paper. But after some time the work is always completed. Still holding the paper on the back *Dromia* may grasp the bridge, which joins the case with the rest of the paper, and tear it. The easier way is to release the case, to turn with the back towards the ground and to finish the work in the same way in which it was begun. It sometimes happens that the crab puts the whole paper on the back without cutting the case. Then the edge of the paper lies at the level of the forehead of the animal and the eyes remain uncovered. Rarely *Dromia* enters under the paper, grasping it near the middle. In both cases after some time the animal always starts the work of cutting a case of suitable size. Ordinarily *Dromia* releases the paper, turns on its back and begins to work as described above. If the first tearing off of the paper occurs in the middle of the whole piece, not at the edge, then *Dromia* folds it twice with the chelæ and tears it on the so-formed new edge. If the paper bears a small hole the crab uses it as the starting point of the work. Less frequent are the cases when *Dromia* keeps its normal position, holding the paper on its back, and without turning begins the cutting. The chelæ tear off one little piece of paper after the other, while the animal slowly revolves under the paper, grasping it every time at a new spot. During

all this the crab retains a firm grasp on the paper. Nearly always *Dromia* works at the middle of one edge of the square, much more rarely the animal cuts off one of its angles. But it is only extremely seldom that the new paper case lies unsymmetrically. It seems probable that the equilibrium plays an important rôle therein.

During two months each of my crabs has been observed several times while working at its new case. There is some evidence that every individual has its own system of working. Four typical modes of cutting may be distinguished. The case is manufactured:

A. In the middle of the longer side of the rectangle.
B. In the middle of the shorter side of the rectangle.
C. At the corner.
D. Somewhere unsymmetrically.

Those four positions are to be seen in Fig. 1. In Table I. I have

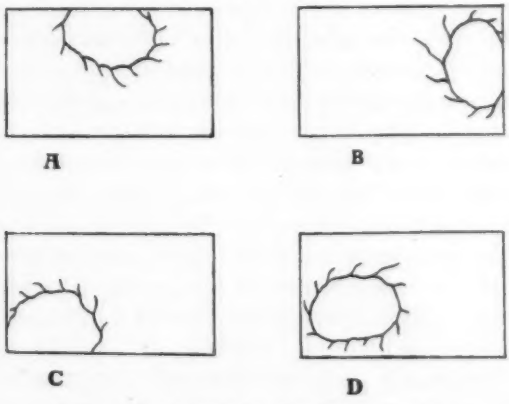


FIG. 1. The four modes of cutting a paper-case.

put together how many times every individual cut the case in each of the above four positions. Only few crabs, as Nos. VII. and IX., did not show any regularity of working. The remaining 8 individuals showed a marked predominance of one mode, especially if we take into consideration that the modes *A* and *B* are very similar.

TABLE I.

	I	II	III	IV	V	VI	VII	VIII	IX	X	Total
A.....	0	10	11	0	9	1	3	0	8	1	43
B.....	3	4	8	2	9	1	6	20	7	16	76
C.....	16	3	0	18	0	14	5	0	3	2	61
D.....	1	3	1	0	2	4	6	0	2	1	20

The letters refer to the four modes of cutting. The Roman numbers designate individuals, the Arabic ones—the number of times for every crab and every category.

Never did the maximum number belong to the category *D*, which means that in 180 cases out of 200 *Dromia* started the work at the middle of one side of the rectangle, or at the corner. Some individuals, as IV., VI., VIII. and X. showed a very striking predominance of one definite mode of cutting and to a certain extent it may be concluded that a definite fashion of working is connected with definite individuals.

Similar modifications of the mode of working were observed when *Dromia* had to cut its case out of a sponge instead of the paper. The sponge was taken from a larger individual and given to a smaller one. The crab always cuts the sponge round and adjusts it to its own size and shape. The sponge is much thicker and less compact, as compared with the paper and therefore the crab was obliged to tear off small pieces of it instead of tearing it as a whole. The pieces, detached from the sponge were then directed towards the mouth where the maxillipedes held them for a while and then let them drop; the current which brings the water to the gill-chamber carried them away. As in the case of paper, *Dromia* may perform its work while lying on its back or in the normal position of the body. The sponge is held tightly by means of 4th–5th pairs of legs. Sometimes the crab will carry on its back a sponge which is much larger than the normal case and will start the work only after a considerable time. But sooner or later the sponge always becomes adjusted to the size of the animal.

A very interesting reaction occurs when we put into the aquarium a large stone covered with a sponge. The animal very soon climbs upon it, grasps the sponge at two points, and tries to tear it off from the stone. After some unsuccessful attempts

it begins to work systematically, tearing off small pieces of sponge along a fairly regular elliptical line, while it turns slowly at the spot. A regular elliptical furrow results, which goes through the whole thickness of the sponge reaching down to the surface of the stone. This furrow is the outline of the future case. Now *Dromia* grasps the periphery of the furrow at two points and pulls it towards itself trying to tear the sponge off. This movement differs very distinctly from the behavior of *Dromia* while cutting the sponge through. As the sponge holds very tightly this attempt usually remains without success. Following this *Dromia* begins again to tear pieces of sponge, but now it works along the furrow trying to press the chelæ between the sponge and the stone. Sometimes the animal proceeds in one direction towards the center of the sponge, remaining at the same spot during the work, but often it detaches the edge of the sponge from the stone along the whole circumference of the furrow. In the former case it starts the work from below, sitting on the future case and directing its head downward. All the time the planes of both chelæ remain parallel to the surface of the stone and the work now is much more a systematical detaching than cutting, as only few pieces of the sponge are now torn off. After some time the crab tries again to tear off the whole case, grasping the edge of it at two points and pulling it towards itself. Usually this second attempt is made at the right time and the animal, holding the sponge tightly, rolls with it from the stone to the bottom of the aquarium. If however, the attempt was premature *Dromia* returns at once to the interrupted work and after some time repeats the same attempt once more. Sooner or later there always results a case corresponding very exactly to the size of the animal.

It seems that a suitable consistency of the sponge, rather than the curvature of the stone corresponding to the curvature of the animal's back, decides the choice of the spot where *Dromia* starts its work. Therefore very often the manufactured case is too flat and it does not fit exactly to the surface of the back. The crab corrects this by pressing the sponge tightly to the back and bending it out. As the tissues of the sponge are fairly plastic, the resulting curvature of the sponge becomes somewhat

fixed after some time and it remains unaltered even if we remove the case from the animal's back. I noticed repeatedly that the coarse inner surface of the new case soon becomes smooth and resembles closely the inner surfaces of natural cases found in the sea. Very probably it depends once more on the mentioned plasticity of the sponge.

If the stone, which we put into the aquarium, was covered by several distinct sponges of different size, but of somewhat rounded circumference *Dromia* did not cut any of them, but chose the most suitable one and tore it off as described above.

It remains not fully explained why larger specimens of *Dromia* usually carry cases on them which cover only the posterior part of the body and are too small. Those sponges have very often uneven edges and generally they fit much worse than the cases of young individuals. I believe that the comparatively small cases result from the difficulty of finding sufficiently large sponges which may be torn off without being damaged. Younger crabs can use sponges which are smaller and therefore younger and more plastic. Perhaps it accounts for the fact that only the cases of small *Dromia* are usually admirably fitted.

Putting of the Sponge on the Back.

After having manufactured a case *Dromia* puts it on the back. This process proved to be an extremely various one, which depends largely on the random position of the sponge.

1. If we take the sponge off from the crab's back and put it in the aquarium with its concavity directed upward, the animal usually returns to it very soon. *Dromia* grasps the sponge from both sides with the fore legs (Fig. 2*A*) and falls on its back, pulling the sponge over it. The sponge, supported by the legs, lies now with its concavity directed downward (Fig. 2*B*). Lying still in this position the animal begins to turn and to move its case in a horizontal plane until the fore edge of it, in the normal position of the animal directed towards the head, points to the abdomen. An ordinary case has an elliptical circumference and its longer axis lies at right angles to the sagittal axis of the body; the hind edge is marked by its greater curvature, while the fore edge is somewhat flattened.

In the next stage of the process the crab grasps the sponge with the hooks of the 4th and 5th pairs of legs and changing the point on which the hooks hold the inner surface of the case as the crab moves it, lifts its abdominal end above the bottom (Fig. 2*C*) until the longitudinal axis of the body lies vertically and the

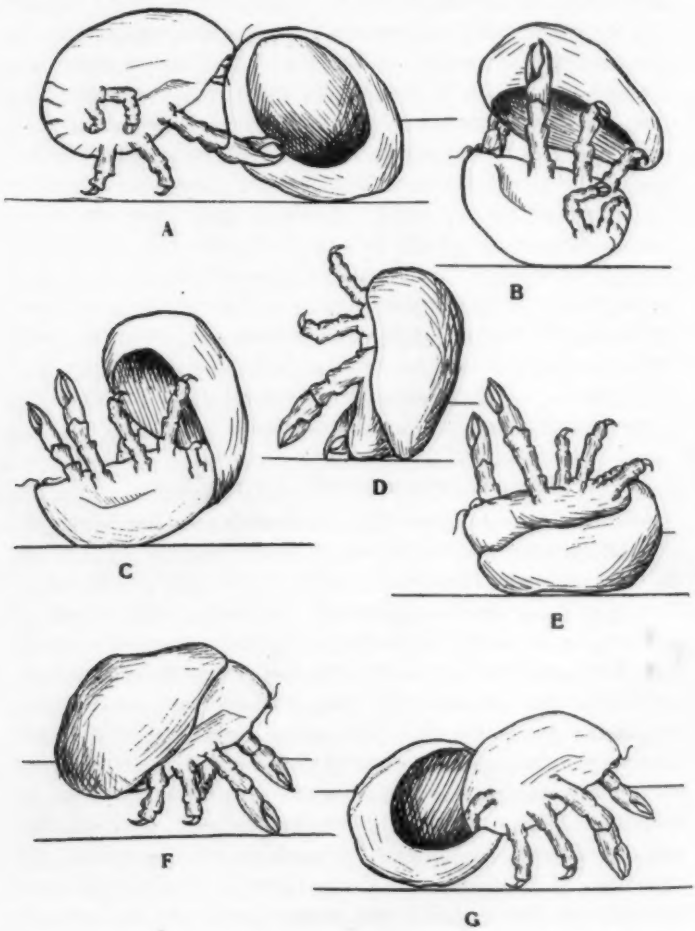


FIG. 2. Different modes of putting the sponge on the back. For explanation see the text.

animal stands on its head (Fig. 2*D*). At the same time the position of the sponge is gradually altered: its fore edge becomes inclined towards the abdomen and the sponge becomes almost vertical in position touching the ground with the fore edge (Fig. 2*D*). As a consequence the abdominal end of the body moves along the concavity of the sponge upwardly and, as the back of the animal remains pressed tightly to the sponge, *Dromia* while standing on its head assumes its normal position towards the sponge. This means that the fore edge of the sponge touches the head. The vertical position of both crab and sponge is of course, a position of an unstable equilibrium, and therefore either the animal falls forward, assuming the normal position, or it falls on its back. In the latter case it can turn in different ways, as described in the paper of Dembowski.

The grade of lifting of the abdomen above the ground may be very different and sometimes the position of the longitudinal axis of the body may be only slightly oblique towards the bottom of the aquarium.

2. In other cases, *Dromia* approaches the sponge, turning its abdominal end to it. The sponge still lies with its cavity directed upwardly. When the abdomen touches the edge the hooks of the 4th and 5th pairs of legs grasp the inner surface of the sponge near its edge (Fig. 2*G*) and then suddenly contracting these legs, the crab simply flings the sponge on the back. In the majority of cases the animal approaches the sponge at the right spot, in others it turns the case correspondingly, either before putting it on the back, or later, in its definite position.

3. There occurs sometimes a modification of case 2. The crab approaches the sponge directing its head towards it, climbs over the sponge and goes farther still in the same direction until the fore end of the body touches the ground on the opposite side of the meeting point (Fig. 2*G*). When only the abdominal end remains in the concavity of the sponge the legs of 4th–5th pairs grasp its inner surface near the edge and fling the sponge on the back.

4. In still other cases *Dromia* enters the sponge (always lying with its concavity directed upwardly) and turns on the back, as shown in Fig. 2*E*. The animal changes its position towards the

sponge several times and at last it always succeeds in assuming the normal one in which the head touches the fore edge of the case. The hooks of the 4th–5th pairs of legs, as usual, grasp the inner surface of the sponge and the animal turns forward to its ordinary position, pulling the sponge behind itself.

In all described cases *Dromia* puts its own sponge on the back in a correct position. But if the sponge belonged to another individual, the position of it becomes often irregular, it may even be put on with its concavity directed upwardly. Still after some time the position will always be corrected.

5. The behavior of the animal changes a little if the sponge is lying with its concavity downward. In the majority of cases it will be grasped with both chelæ and, as before, the animal, falling on its back, pulls the sponge over it. Now the crab turns the sponge not only in a horizontal plane, but holding it with 1st–2d pairs of legs it turns its concavity downwardly. After this the behavior becomes similar to the case 1.

6. *Dromia* may press the abdominal end of the body between the sponge and the ground (the concavity of the sponge being turned downward), then it enters under the sponge and grasps its inner surface with the hooks of the 4th–5th legs. The sponge becomes adjusted at once, or it may be turned correspondingly in a horizontal plane.

7. Before attempting to put the sponge on its back the animal may turn its concavity upward and then it behaves as usual. This turning occurs while *Dromia* pushes the edge of the case with the forehead, or it presses the chelæ between the edge of the sponge and the bottom. If there are some small stones on the ground the task becomes easier, as the stones prevent the gliding of the sponge along the bottom, furnishing at the same time a certain point of support for the animal.

Dromia is able to surmount many unexpected difficulties when putting the case on. If we put a few stones in the sponge, which lies with its concavity directed upward, the sponge becomes too heavy and the animal cannot turn it. After some time however the sponge always will be liberated from the stones. In most cases *Dromia* attempts at first one of the described above modes of acting, as if there were no difficulty at all. But as those

attempts will be unsuccessful now, the animal presses its fore or hind end between the sponge and the ground until it overthrows the case and causes the stones to fall out. We have seen already that the crab behaves sometimes in that way when the sponge lies with its concavity downward. It will be noted, however, that the animal never does this when the sponge points with its concavity upward and there is no doubt that this behavior is caused by the impossibility of using one of the ordinary ways.

In other cases the stones will be pushed with one chela towards the edge of the sponge until they fall out. This work is but seldom carried to an end and some of the stones usually remain when the animal puts the sponge on its back. The remaining stones prevent the proper adjustment of the sponge and after some time *Dromia* repeatedly lifts the sponge over its back and presses it again, which finally causes the stones to fall out, slipping between the back of the animal and the inner surface of the sponge.

Dromia can manage the liberating even in cases when the sponge remains completely covered by the stones and invisible. This requires however some previous learning. Every time that the crab succeeds in putting the sponge on the back, we take it off and put still more and more stones into it, keeping the sponge always at the same point of the bottom. After some time *Dromia* becomes accustomed to the place where the sponge lies and it goes directly towards it, trying to move the stones and liberate the sponge. This behavior becomes striking when, after several repetitions, we put the sponge in quite a different place. The animal goes nevertheless directly to the previous spot and begins to seek the sponge. As control experiments showed, it never behaves in this way without previous learning.

In another series of experiments I fastened the sponge to a little wire hook. In the meantime the crab manufactured a new case of paper. In order to make the sponge more conspicuous I chose a red one and put it into a porcelain dish, hanging it so high on the wall that the crab could hardly reach it. When I put the crab in, it directed itself at once towards the sponge, *dropping the paper case on the way.* With some difficulty the animal succeeded in grasping the hanging sponge with the chelæ.

It then climbed over the sponge, very soon found the wire hook, and began to tear off small pieces of sponge around it. After a short time the sponge became liberated and the *Dromia* rolled down with it, and put the sponge on the back. From the moment of putting the crab into the vessel to the liberation of the sponge 7 minutes elapsed. I repeated this experiment several times and I found that the described behavior is a typical one. Shortly after liberating the sponge I took it off again, giving to the animal the paper case which was accepted. Then the sponge was again attached very tightly to a wire hook and was hung on the wall. After being put into the vessel, the crab once more directed itself immediately towards the sponge, climbed over it and only now dropped the paper case. The next moment it adjusted itself in the sponge, grasping its inner surface with the 4th–5th pairs of legs and pressing the back to the concavity of the case, assuming its normal position. Now the animal tried to go with the sponge, but naturally succeeded only in gliding with the legs on the smooth wall of the vessel. Then it changed several times its position in the sponge, still holding it tightly, but only after two hours of continuous attempts it found the wire hook, with the chelæ. Now the liberation took only few minutes and as before *Dromia* rolled with the sponge to the bottom. I repeated this experiment with the same individual over 60 times in about 20 days and found that in the great majority of cases the animal directs itself immediately to the wire hook and only very rarely, and during a short time, it tries to go with the sponge on the back. The time of liberating varied from 6 to 20 minutes, on the average it was 8 minutes. Many other individuals showed exactly the same reactions. After some time, ordinarily after a few minutes, each crab succeeded in finding the wire hook and liberating the sponge.

It is to be noticed that *Dromia* possesses a marked faculty of choosing the material for its case, which depends somewhat on the past of the animal. Several facts corroborate this conclusion.

1. We take off the sponge from a fresh *Dromia* and we put into the aquarium several different materials, such as a sponge, an artificial case made of plastelin, a piece of rag, paper, etc. Under those conditions, the animal chooses the sponge. In most cases it recognizes its own case from many other sponges.

2. If the crab has carried an artificial plastelin case on its back for a long time, it often happens that it chooses its own plastelin case, even if near to it there is a sponge of suitable size.

3. *Dromia* may be found without any case at all. Such an animal always chooses a sponge, and so does an individual which remained in the aquarium uncovered for a certain time.

4. We give cases of different kinds to an individual, which carries its own case on the back. If the latter was a paper case, then *Dromia* always drops it and chooses a sponge. Under the same conditions a case of plasteline was dropped only sometimes, but a normal case of sponge was nearly always kept by the animal.

Conclusions.

To a certain extent the described facts furnish an answer to the questions raised at the beginning of this paper. It follows certainly from them that there is no reason to speak about symbiosis of *Dromia* and sponge, as the mutual adaptation is entirely lacking. If the crab is especially adapted to carry a case on its back, the sponge surely remains completely passive. The lack of any specificity of the symbiosis proves it very clearly. It is not plain what may be the use of the sponge to *Dromia*. Polimanti put covered and uncovered *Dromia* into an aquarium containing an *Octopus*. He found that the uncovered individuals were devoured, just as any crabs of some other species, but the covered ones remained safe. However Polimanti does not decide whether the *Octopus* simply was unable to see the covered animals, or whether the sponge held it away. At all events, as far as the optical side of the problem is concerned, it may be interpreted in different ways. In most cases there is no correspondence in color between the sponge and the surroundings. The sponge, which *Dromia* carries on it, may be of very different, often bright, colors: red, yellow, purple, etc. The presence of the sponge even facilitates the catching of *Dromia*, as the crab usually remains on vertical walls very near to the surface of the sea. An uncovered specimen is less conspicuous by far, on account of its uniform gray or brownish coloration. But just as frequently the sponge may be of a somewhat protective coloration, gray, brownish, or greenish, which contradicts again the theory of a

warning color. It will be safer probably to conclude that the color of the sponge is an accidental one and that it does not serve any special purpose.

The conformity of the inner surface of the sponge and of the back of *Dromia*, especially in young individuals, may be explained without any artificial assumptions, like the theories of Renier and Vosmaer. We did see that the animal possesses the faculty of cutting a sponge of suitable size and circumference, tearing it off from a stone, and putting it on its back. As the sponge remains then pressed tightly to the back of the crab, it soon assumes a suitable shape. When moulting the crab must drop the sponge. But we know that all the crabs after the moult remain nearly motionless until the new chitin hardens. We know moreover, that *Dromia* can find a sponge which is entirely covered by stones, and that it can dig it out. It follows therefrom, that very often at least after the moult the crab may pick up its own sponge again. But still we do not know exactly if the rapidity of growth is the same in both organisms. At all events it must happen very often that under its natural life conditions *Dromia* is compelled to find a new case for itself.

The described facts confirm once more the very complex character of reactions of a crab. The behavior of the animal towards the case is a series of most complicated movements, too complicated in fact to be explained by simple external stimulations. From the physiological point of view there exists the possibility that the pressure of the case on the sensory hairs of the animal's back and the effect of its weight on the 4th–5th pairs of ambulatory legs form the stimulus which inhibits the movements leading to the finding and manufacturing a new case. Conversely, the lack of such stimulus may be one of the causes of those movements. But there exists an interesting fact that contradicts the above assumption. One individual, while carrying on its back its normal sponge case, began to manufacture a new case from a piece of paper, without dropping its sponge. Such facts are rare, it is true. However, it happens more frequently that individuals, which are carrying a case of plasteline or paper, choose a sponge and grasp it with the chelæ, and only now drop their cases from the back. This behavior

may be called "senseless," but it proves that the stimulus, which compels the animal to act in a definite way is a complex one and that the optical side of the question plays an important rôle therein. We did see, in fact, that the crab rushes at once towards a distant sponge, if it is made conspicuous by putting it into a porcelain dish.

As in all the reactions of a crab, its behavior towards the sponge varies from individual to individual. In some cases, but not always, it may depend on a different activity of different individuals. There are marked differences, depending on the age. Older specimens are by far less active, and this bears on the whole behavior. The young crabs begin their work of cutting the case much sooner, the work itself is done more quickly, and the cases are made more carefully. They attack very often other individuals and take their cases away, which occurs only rarely in larger crabs. Those observations agree with the statement of Watson. This author found, in fact, that young rats, 23 days of age, possess already the faculty of performing all the tasks that a rat is able to perform, in spite of their nervous system being not yet definitely developed. On account of their greater activity, the young rats often solve different problems more quickly than the adult individuals.

Some facts reported above confirm the conclusions of Yerkes, Yerkes and Huggins, Spaulding, v.d. Heyde, Mikhailoff, concerning the existence of associative memory in the crab. If a crab carried for a long time a case of sponge or plasteline, and if this case be taken off from it, the animal nearly always chooses from several cases its previous one. The crab may learn to come to a definite spot in the aquarium, where its sponge is lying under the stones, even if this sponge be put somewhere else.

The reported observations are somewhat fragmentary. The problem of the conformity of shape of both organisms is not entirely solved by them, as we do not know what may be the behavior of very young crabs that are putting on a sponge for the first time. The behavior after the moult also has not yet been observed. Nevertheless the described facts do elucidate some points of the question and, which is perhaps more important, they show many experimental possibilities of studying the behavior of this most interesting animal.

LITERATURE CITED.

1. **Celesia P.**

'93 Della Suberites domuncula e della sua simbiozi con paguri. Atti Soc. Ligust. Sc. nat geogr. Vol. 4.

2. **Dembowski J.**

'25 On the Reaction of Turning in *Dromia vulgaris*. Prace Inst. im. M. Nenckiego, Warsaw (Polish).

3. **Heyde v. d. H.**

'20 Über die Lernfähigkeit der Strandkrabbe (*Carcinus mænas*). Biolog. Zentralbl., Bd. 40.

4. **Mikhailoff S.**

'22 Expériences réflexologiques. Bull. de l'Inst. Océanograph. de Monaco, No. 375 et No. 418.

5. **Polimanti O.**

'11 Studi di fisiologia etiologica. I. Zoölog. Jahrb. allgem. Zoöl., Bd 30.

6. **Renier M.**

1807 Tavole per servire alla classificazione degli animali. (Cited after Polimanti, l. c.)

7. **Spaulding E.**

'06 An Establishment of Association in Hermit Crabs. Journ. of Comp. Neurol. and Psychol., Vol. XIV.

8. **Topsent E.**

'00 Étude monographique des spongiaires de France. Arch de Zoöl. expériment, et gén., S. III, t. 8.

9. **Vosmaer G. C. J.**

'87 Spongien. Bronn's Klassen u. Ordn. d. Tierreichs.

10. **Watson J. B.**

'03 An Experimental Study on the Psychical Development of the White Rat Correlated with the Growth of its Nervous System. Chicago.

11. **Yerkes R.**

'02 Habit Formation in the Green Crab, *Carcinus granulatus*. BIOL. BULL., Vol. 3.

12. **Yerkes R. and Huggins G. E.**

'09 Habit Formation in the Crawfish *Cambarus affinis*. Harvard Psych. Studies, Vol. I.